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THE SYNTHESIS OF FAT FROM PROTEIN BY THE ALBINO RAT

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(Received for publication June 27, 1939)

Whether protein may be transformed into fat by higher animals has long been a question of particular interest to students of nutrition. The generally accepted view has been that such a transformation may take place. Protein and certain amino acids yield considerable proportions of dextrose when fed to diabetics or to animals which have been depancreatized or treated with phlorizin. Since dextrose from other sources is readily changed into fat in the animal body, it is reasonable to expect that dextrose derived from protein also may be so transformed. However, Longenecker ('39) appears to have been the first to present direct experimental evidence that normal higher animals can synthesize fat from protein. In his experiments, male albino rats were grown on an adequate stock diet to weights of about 260 gm. They were next fasted until they had lost 27 to 28% in weight. The carcasses of some, after rejection of certain portions, were then analyzed for fat content. The remaining rats were transferred to a high-protein ration until they had practically recovered their former weights, at which time their carcasses were similarly analyzed for fat content. Longenecker reported that more than one-third of the gain in weight of the second group was due to the deposition of fat, a quantity representing twenty to thirty times the amount of lipids contained in the total ration consumed during the refeeding period.

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The present writers had completed the experiments herein described and had practically completed their descriptive manuscript before Longenecker's paper appeared. Though their results now become merely corroborative, nevertheless the data are of interest because the problem was attacked by a method which differed in some details from that employed by Longenecker. The purpose of the present experiments was to determine whether young albino rats could materially increase their store of fat when fed a diet consisting chiefly of casein and practically free from fat and carbohydrates.

PREPARATION OF DIET

The fat- and carbohydrate-free diet contained purified casein, 94%; yeast vitamins, 2%; salt mixture, 4%; plus vitamins A and D equivalent to 2% of cod liver oil, added in the form of an ether extract of the saponified oil. The ingredients were prepared as follows:

Commercial casein was extracted with ethyl ether by percolation until practically free from ether extract and was then treated with successive portions of boiling 95% alcohol on a steam bath until practically free from alcohol-soluble material. Analysis of the purified casein by digestion with 30% potassium hydroxide solution, acidification, and extraction with petroleum ether showed practically no fatty acids. Water-soluble vitamins were supplied in the form of commercial concentrate from brewers' yeast, which yielded only a trace of fatty acids when analyzed by the above method. Reducing sugar was not found in either the casein or the yeast vitamins. The salt mixture was that regularly employed in feeding experiments with rats in this laboratory.

DETERMINATION OF TOTAL FATTY ACIDS IN RATS

Each entire carcass was frozen hard at —9°C., chopped into pieces, and passed several times through a meat grinder. Approximately 10 gm. of the ground material was weighed into

a 150-cc. beaker, 40 cc. of 30% potassium hydroxide solution was added, and the mixture was heated on a steam bath until the soft tissues were dissolved. Ethyl alcohol was added and heating was continued until saponification was completed. The material was transferred to a separatory funnel and acidified with hydrochloric acid. When cool, the mixture was extracted with two successive 100 cc. portions of petroleum ether. The ether extract was washed with successive portions of water until free from hydrochloric acid, transferred to an Erlenmeyer flask and the ether was expelled on a steam bath. The fatty acids were dried to constant weight at 100°C.

FEEDING EXPERIMENTS

Two closely similar male albino rats were selected from each of seven litters. One rat of each litter pair was chloroformed at the beginning of the experiment, frozen, ground, and analyzed for total fatty acid, with inclusion of intestinal contents. The other rat of each pair was fed the fat- and carbohydrate-free diet for 60 days and was then likewise chloroformed, frozen and analyzed for total fatty acid. During the feeding period each rat was kept in an individual cage with a raised screen bottom, and was supplied with feed in a self-feeder, as well as with water. The rats were weighed twice weekly and the quantity of feed eaten was recorded. The results of this experiment are shown in table 1 and for comparison the results obtained with a group of rats fed a diet containing 5% of lard are included.

All rats fed the fat- and carbohydrate-free diet developed pronounced symptoms of linoleic acid deficiency before the end of the experiment, the first symptom appearing after about 30 days. All these rats grew very slowly and appeared poorly nourished. Table 1 shows that the rats fed the fat- and carbohydrate-free diet made an average daily gain in weight of only approximately 1 gm. as compared with a gain of 3.8 gm. by the rats fed the diet containing 5% lard. The latter group of rats also utilized their feed much more efficiently.

TABLE 1

Growth in 60 days of male rats fed a fat- and carbohydrate-free diet compared with growth of male rats fed a diet containing lard and dextrose

DESCRIPTION OF DIET		RAT NO.	LITTER NO.	INITIAL WEIGHT	GAIN IN WEIGHT	FEED CONSUMED	ENERGY INTAKE	GAIN IN WEIGHT PER 100 CAL.
Ingredient	%			gm.	gm.	gm.	Cal.	gm.
Casein	94	2	60C	45	89	443	1595	5.58
Yeast vitamins	2	4	64B	39	47	360	1296	3.63
Salt mixture	4	6	66A	45	64	368	1325	4.83
Vitamins A, D	Added	8	67B	40	40	352	1267	3.16
		10	67D	40	83	476	1714	4.84
		12	70A	44	73	447	1609	4.54
		14	70C	41	73	455	1638	4.46
Average				42	67	414	1493	4.43
Refined lard	5.0	1359A	59C	39	171	679	2444	7.00
Casein	20.0	1359B	61B	40	258	864	3110	8.30
Yeast vitamins	2.0	1359C	62A	40	247	833	2999	8.24
Salt mixture	4.3	1359D	62D	39	251	870	3132	8.01
Dextrose	68.7	1359E	63C	43	210	769	2768	7.59
Vitamins A, D	Added	1359F	64C	40	229	838	3017	7.59
		1359G	65D	38	231	839	3020	7.65
		1359H	66B	38	233	790	2844	8.19
Average				40	229	810	2917	7.82

STORAGE OF FATTY ACIDS BY RATS FED A FAT- AND CARBOHYDRATE-FREE DIET

In table 2 is shown the total quantity of fatty acids in the carcass of each rat at the end of the 60-day experiment in comparison with the quantity present in its litter mate at the beginning of the experiment, correlation being made for differences in the initial weights of the rats.

The results of these experiments show that each of seven male albino rats fed a fat- and carbohydrate-free diet for 60 days contained much more fatty acid at the end of the experiment than did its litter mate at the beginning. The increase ranged from 38 to 358% with an average of 257%, and the total increase for the seven rats amounted to 47.92 gm. of fatty acid. More than one-tenth of the gain in weight was constituted by fatty acids thus derived, which represents a considerably larger proportion of lipids. These figures do

not include fatty acids excreted in the feces or those oxidized by the rats. Hence, the above noted increases in fatty acid represent minimum quantities synthesized. In view of the severe conditions under which these experiments were conducted, particularly the lack of linoleic acid in the diet, the results show conclusively that the albino rat can synthesize fat from protein.

TABLE 2

Change in fatty-acid content of male albino rats when fed a fat- and carbohydrate-free diet

			AT BEGINNING OF EXPERIMENT			AT END OF 60-DAY EXPERIMENT			
RAT NO.	LITTER NO.	AGE	Initial weight	Total fatty acids		Gain in weight	Total fatty acids	Gain in fatty acids	
				Determined	Estimated				
		days	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*%*
1	60C	21	42	2.62					
2	60C	21	45		2.81	89	11.72	8.91	317
3	64B	20	38	2.53					
4	64B	20	39		2.60	47	9.65	7.05	271
5	66A	21	45	2.61					
6	66A	21	45		2.61	64	11.66	9.05	347
7	67B	21	38	2.96					
8	67B	21	40		3.12	40	4.29	1.17	38
9	67D	21	38	3.40					
10	67D	21	40		3.58	83	8.25	4.67	130
11	70A	25	42	2.86					
12	70A	25	44		2.99	73	13.11	10.12	338
13	70C	22	41	1.94					
14	70C	22	41		1.94	73	8.89	6.95	358
	Average				2.81	67	9.65	6.85	257
	Total				19.65		67.57	47.92	

SUMMARY AND CONCLUSIONS

Young male albino rats were fed a diet consisting chiefly of casein, but free from fat and carbohydrates, for 60 days in order to determine whether the rat can synthesize fat from protein. Two similar rats were selected from each of seven litters. One rat of each pair was analyzed for total fatty acid at the beginning of the experiment; its litter mate was analyzed at the end. Each rat fed the test diet for 60 days was

found to contain a significantly larger quantity of fatty acid than had been present in its litter mate at the beginning of the experiment. The increases in fatty acid ranged from 38 to 358%, with an average of 257%. The total quantity of fatty acid in the seven rats at the end of the experiment was greater by 47.92 gm. than the total quantity in the seven litter mates at the beginning of the experiment.

The results of these experiments show conclusively that the albino rat can synthesize fat from protein, in corroboration of Longenecker's report.

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THE INFLUENCE OF A CARCINOGENIC COMPOUND ON THE HEPATIC STORAGE OF VITAMINS

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(Received for publication June 12, 1939)

INTRODUCTION

The discovery made by Cook and his associates ('32) that certain anthracene derivatives possess the property of producing malignant tumors in animals has given rise to interesting work on the biological and chemical changes brought about by their presence in the tissues. The action of a typical carcinogenic compound, 1,2,5,6-dibenzanthracene, on mitochondria has been described by Goerner ('37–'38). Its effect on the lipid composition of these cytoplasmic structures led to a study of its influence both on the hepatic storage of the fat-soluble vitamins A and D and on the related processes of growth and calcification. In contrast with any effect that dibenzanthracene might exert on the hepatic storage of these two vitamins, its action on a third compound possessing no lipid properties was determined, namely, vitamin C.

METHODS

The two types of animals selected for use in the experiments were divided into three groups each made up of thirty-six rats and eighteen rabbits. The treated group was injected intraperitoneally with 0.1% colloidal 1,2,5,6-dibenzanthracene in 1% gelatin solution. Each rat received 3 mg. and each rabbit 10 mg. of the hydrocarbon per week. The control group of animals was similarly injected with 1% gelatin solution alone, and the normal group remained untreated. The gelatin

solutions were warmed before injection to liquefy them. The initial weights of the rats ranged from 70 to 100 gm., and of the rabbits from 1000 to 1350 gm. All were fed a commercial chow that insured normal growth and maintenance of health of each type of animal.

At various intervals animals of each group were sacrificed and the determination of vitamins in the livers immediately begun. Vitamin A was extracted according to the procedure described by Moore ('30). The ether extract obtained was then evaporated, the fatty residue dissolved in chloroform and the vitamin A determined colorimetrically with the use of a saturated solution of antimony trichloride in chloroform according to the method devised by Rosenthal and Erdélyi ('33), and modified by Andersen and Levine ('35). A biologically standardized preparation of vitamin A containing 55,000 U.S.P. units per gram was used as a standard for comparison after suitable dilution with chloroform.

To determine the effect of the hydrocarbon on the storage of vitamin C, a part of the liver from each of the above animals was set aside for its estimation as described by Birch, Harris and Ray ('33). Instead of trichloracetic acid to extract the vitamin from the liver tissues, a 2% solution of metaphosphoric acid was employed as suggested by Fujita and Iwatake ('35) to prevent deterioration of the vitamin. The acid extract was then titrated against a solution of 2,6-dichlorophenolindophenol which had been previously standardized against ascorbic acid.

The determination of vitamin D in the liver was carried out by means of biological assay described in the United States Pharmacopoeia XI ('35). Young rats of approximately the same age and weighing from 35 to 45 gm. were placed on the standard rachitogenic diet for a period of 3 weeks. During this time extracts were prepared from the livers of both rats and rabbits which had been treated with various amounts of dibenzanthracene as described above. Extracts were also prepared from the livers of the control animals which had received injections of 1% gelatin alone and of the normal

untreated animals. All extracts were made in accordance with the procedure given by Heyman ('37). The fatty residues from weighed portions of liver obtained by evaporation of the ether extracts were then made up into various dilutions with olive oil. These liver assay oils containing different concentrations of the fatty residue were fed in 0.1 cc. portions daily for 8 days to the weighed rachitic rats. Vitamin D in the form of a biologically standardized preparation was given to another group of rachitic animals for the same period, these serving as the standard reference group. A third group of rachitic rats received 0.1 cc. of plain olive oil for the same period. These gave evidence of severe rickets at the end of the test and served to show the absence of vitamin D in the olive oil used for dilutions of the fatty residues of liver extracts mentioned above. A fourth group of rachitic rats was kept throughout the assay period on the rachitogenic diet and received no other treatment. The severe rickets which these animals showed at the end of the test period indicated the persistence of the rachitic process in the untreated rats. All animals were kept in the dark during the preparation and assay periods.

From the daily dose of the biologically standardized preparation of vitamin D which was just sufficient to cause definite healing of rickets as shown by the 'line test,' the vitamin D content of the dilution of liver assay oil necessary to bring about a similar evidence of healing may be calculated. From this result the concentration of vitamin D per 100 gm. whole liver may be obtained.

In order to study the effect of dibenzanthracene on growth and calcification, a group of young rats weighing from 45 to 50 gm. was injected intraperitoneally each week for 3 months with 3 mg. of the hydrocarbon. A control group received injections of 1% gelatin and a third or normal group remained untreated. The thirty animals of each group were weighed daily at first and then twice a week during the remainder of the experiment. Each week an animal of each group was sacrificed and the hepatic vitamin A determined as described

above. The effect of the hydrocarbon on calcification was also studied on these animals by analysis of the leg bones according to the procedure described by Chick, Korenchevsky and Roscoe ('26). All animals were fed a standard commercial chow.

RESULTS

The livers of both rats and rabbits treated with dibenzanthracene were greatly enlarged, weighing from two to three times that of the normal animals. All showed macroscopic signs of marked fatty changes in the organ as first reported by Polson ('36). This, as well as the microscopic evidence of fatty degeneration and necrosis, indicated the severity of tissue damage brought about by the hydrocarbon. One might expect to find, under these circumstances, that the hepatic storage of both fat-soluble vitamins would be affected. As the results in table 1 show, the storage of vitamin A was decreased to a great extent by dibenzanthracene while that of vitamin D remained unchanged. Extraction of the alkalinized urine of the treated animals failed to show the presence of any vitamin A. The vitamin C content of the treated rats and rabbits was entirely comparable to that of the respective normal animals.

Results of the experiments on the influence of dibenzanthracene on the growth of young rats showed that the gain in weight of the treated group at the end of 1 month was only 70% that of the normal; at the end of 2 months, 60.7%; and at the end of 3 months, it had fallen to as low as 48.4%. Analysis of the liver tissues made each week showed a steadily decreasing vitamin A content in the treated group as compared with that of the normal rats. Inflammatory processes of the conjunctiva indicative of xerophthalmia were also observed in the treated rats during the latter part of the experiment. At the end of 3.5 months all the treated animals that had not been used for analytical purposes had died, while only one death occurred in the normal group. The increasing inhibition of growth caused by the injections of dibenzan-

thracene was parallelled by the progressive decrease of hepatic vitamin A resulting from damage to the liver tissues by the hydrocarbon. The effect on the storage of this vitamin was especially noticeable in those animals which had received a large total dose of dibenzanthracene. In such instances, vitamin A was entirely absent from the liver tissues, even though the content of vitamins C and D was normal.

The young rats used in the growth experiment served also to determine the influence of the hydrocarbon on calcification of osseous tissues. The results of these tests showed that the

TABLE 1

Effect of dibenzanthracene on storage of vitamins A, C and D

ANIMALS	MEAN, MINIMUM AND MAXIMUM VALUES			
	Total dose of dibenzanthracene given	Vitamin A per 100 gm. liver	Vitamin C per 100 gm. liver	Vitamin D per 100 gm. liver[2]
	mg.	*U.S.P. units*	*mg.*	*U.S.P. units*
Rats				
Normal (36)[1]	0	5884 (422–12,650)	26.8 (19.8–36.1)	37 (32–56)
Control (36)	0	6452 (516–13,574)	28.9 (20.0–33.8)	28 (21–45)
Treated (32)	30.1 (9–45)	28 (0–160)	28.3 (20.8–34.1)	32 (26–52)
Rabbits				
Normal (18)	0	2810 (914–4,265)	10.4 (7.4–17.1)	14 (11–18)
Control (18)	0	3188 (1096–5,274)	10.2 (7.0–16.4)	12 (8–20)
Treated (17)	72.2 (20–130)	36 (0–380)	11.0 (7.2–17.9)	10 (9–16)

[1] Numbers in parentheses indicate the number of animals observed.

[2] These vitamin D data pertain to groups of six animals.

ratio of mineral ash (A) to fat and organic material (R) ranged from 1.0 to 1.4 in the treated group as compared with an A/R ratio of 1.0 to 1.5 in the normal animals. In the treated rats the per cent of bone ash in the dry fat-free bones varied from 51.6 to 57.8 with an average of 55.3; in the normal animals, these values ranged from 52.6 to 56.8%, with an average of 54.8. The results of these experiments served to confirm those on vitamin D previously obtained, in which it was shown that dibenzanthracene had little effect on the storage of this compound in the liver.

The gelatin injections in the control group of animals did not affect the storage of any of the three vitamins, the rate of growth or the process of calcification.

SUMMARY

1. Intraperitoneal injections of 1,2,5,6-dibenzanthracene decreased the hepatic storage of vitamin A but not that of vitamins C or D. No vitamin A could be detected, however, in the urine excreted by the treated animals.

2. The inhibition of growth caused by dibenzanthracene in young rats was paralleled by the decrease in hepatic vitamin A.

3. No effect on the calcification of osseous tissues was observed in young rats treated with the hydrocarbon.

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THE INFLUENCE OF ARSENIC AND CERTAIN OTHER ELEMENTS ON THE TOXICITY OF SELENIFEROUS GRAINS [1, 2]

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THREE FIGURES

(Received for publication June 12, 1939)

Early investigations of the 'alkali disease' revealed that arsenic, vanadium, molybdenum, and chromium as well as selenium are present in some of the soils, feeds, and waters from the toxic areas (Beath et al., '35; Byers, '34; Miller and Byers, '35). Later studies on the comparative toxicities of selenium, tellurium, arsenic, molybdenum, tungsten, and vanadium (Franke and Moxon, '37) revealed that selenium was the only one of these elements to cause a severe pathological condition of the liver.

Since some of these elements occur with selenium in nature, it was of interest to determine what effect they might have on the toxicity of seleniferous grains. As a result of this study a method of counteracting selenium toxicity in laboratory animals has been developed.

EXPERIMENTAL

Sixty-five rats were divided into thirteen groups of five rats each. The groups were balanced as nearly as possible

[1] Published with the permission of the director of the Agricultural Experiment Station of South Dakota State College, Brookings, South Dakota.

[2] A preliminary report on part of this work was presented at the 96th Meeting of the American Chemical Society (Milwaukee, September, 1938) and also in Science, vol. 88, p. 81, 1938.

as regards weight and sex. The first twelve groups were fed a diet composed of:

	%
Seleniferous wheat (14 p.p.m. selenium)	82
Casein	10
Lard	3
Dehydrated yeast (Northwestern)	2
Cod liver oil	2
McCollum's salt mixture	1
	100

The diet contained 11 p.p.m selenium by analysis.

Each of eleven of the first twelve groups was given 5.0 parts per million (p.p.m.) of the following elements in their drinking water: tungsten, fluorine, molybdenum, arsenic, chromium, vanadium, cadmium, zinc, cobalt, uranium and nickel. An amount of a water-soluble salt (table 1) equivalent to 5.0 p.p.m. of each element was given in distilled water. Group no. 12 received distilled water without an added element. The thirteenth group, which served as a positive control, received distilled water and a diet similar to the one above except that control or selenium-free wheat was used in place of the seleniferous wheat.

Differences in weight and food consumption among the various groups were noted soon after the beginning of the experiment. Since the results were very significant at the end of 60 days a summary of the condition of the rats in the various groups at this time is included in table 1. At this stage of the experiment it appeared that fluorine, molybdenum, chromium, vanadium, cadmium, zinc, cobalt, and uranium caused an increase in the mortality rate. Since there were no controls which received these elements in the absence of selenium we cannot state whether the increased mortality rate was due to the element in the water or to the combined effect of selenium and the element. Very significant, however, was the fact that all rats in the tungsten and arsenic groups were living and normal in appearance at the end of 60 days.

At the end of 130 days the survivors (table 1) were killed and examined for liver damage. Those rats fed selenium

TABLE 1

GROUP	ELEMENT	AMOUNT	SALT USED	AVERAGE WEIGHT OF SURVIVORS AT 60 DAYS	SUMMARY AT 60 DAYS	AVERAGE WEIGHT OF SURVIVORS AT 130 DAYS	CONDITION OF SURVIVORS AT 130 DAYS
		p.p.m.		gms.			
I	W	5	$Na_2WO_4 \cdot 2H_2O$	70	All rats were living.	90	1 living (normal)
II	F	5	NaF	74	Three rats were dead at the end of 35 days. All showed necrosis of the livers, severe atrophy of central liver lobes, adhesions of liver to diaphragm and peritoneum, and internal hemorrhages.		All dead
III	Mo	5	$(NH_4)_2MoO_4 \cdot 2HOH$	107	Four rats dead at end of 40 days. All showed hemorrhages, atrophy and necrosis of livers.		All dead
IV	As	5	Na_2HAsO_3	115	Normal appearance. All living.	195	4 living; appear normal
V	Cr	5	$(NH_4)_2Cr_2O_7$	75	Four rats dead at end of 45 days. All had atrophy of central liver lobes and some showed necrosis of livers.		All dead
VI	V	5	$NaVO_3$	68	Four rats dead at end of 45 days. All had atrophy of central liver lobes, and necrotic and hemorrhagic livers.	132	1 living; rough coat
VII	Cd	5	$CdCl_2 \cdot 2\ 1/2\ H_2O$	106	Four rats were dead at end of 35 days. They all had necrosis and atrophy of the central liver lobes.		All dead
VIII	Zn	5	$ZnCl_2$		All five rats were dead at the end of 50 days. They had atrophy of the central liver lobes, severe necrosis, and hemachromatosis of the livers.		
IX	Co	5	$CoCl_2$		All five rats dead by the end of 35 days. They had atrophy of the central liver lobes and some necrosis of the livers.		
X	U	5	$UO_2(C_2H_3O_2)_2 \cdot 2H_2O$	103	Four rats were dead at end of 40 days. They had atrophy of central liver lobes and necrosis of the livers.		All dead
XI	Ni	5	$NiCl_2$	112	Two were dead at the end of 55 days. Both had necrosis and hemachromatosis of the livers, and atrophy of central liver lobes.	169	2 living; inactive; rough coat
XII	Se diet and distilled water			120	One rat died at the end of 45 days. It had internal hemorrhages, atrophy of central liver lobe, and hemachromatosis of the liver.	141	3 living; loss of hair; inactive
XIII	Control feed and distilled water			132	All rats normal.	201	All living and normal

alone, selenium with nickel, and the one survivor receiving selenium with vanadium, all had atrophied and necrotic livers, some of which exhibited hemachromatosis. The livers of the one survivor in the tungsten group, those fed arsenic with selenium, and the controls were normal in appearance. The livers were dried and analyzed for selenium. The results of the analyses together with the average total food intake, average total selenium intake and gain in weight of the survivors in each group are presented in table 2.

TABLE 2

GROUP	ELEMENT IN WATER	NUMBER SURVIVING AT 130 DAYS	AVERAGE GAIN PER RAT FOR 130-DAY PERIOD (survivors)	AVERAGE TOTAL FOOD INTAKE OF SURVIVORS	AVERAGE TOTAL Se INTAKE OF SURVIVORS	AVERAGE Se CONTENT OF LIVER p.p.m. dry weight	CONDITION OF LIVER
			gm.	*gm.*	*mg.*		
I	W	1	57	666	7.2	66	Normal appearance
IV	As	4	163.5	1277	14.2	26.8	Normal appearance
VI	V	1	99	1313	14.4	30	Atrophied Necrotic
XI	Ni	2	135	1207	13.2	52	Atrophied
XII	None	3	109	1032	11.3	45.5	Atrophied Necrotic Hemochromatosis
XIII	None	5	179	1389	None	0.0	Normal

Although the selenium-arsenic group consumed more of the diet and consequently had a greater intake of selenium, the concentration of this element in their livers was much lower than in the other groups receiving the seleniferous diet, with one exception. The one surviving rat in the group receiving vanadium had the highest selenium intake but had a relatively low concentration of selenium in the liver. However, all of the rats in the selenium-vanadium group showed typical symptoms of selenium poisoning.

The four rats in the tungsten group which did not survive the 130-day period died very near the end of the trial and at

autopsy none showed any appreciable liver damage. Also the one remaining rat of the group showed no gross liver damage although the liver did contain a high concentration of selenium. Group IV, those receiving arsenic in their water, grew normally, had good appetites and showed no liver damage when examined at the end of the experiment.

Since it appeared that tungsten and arsenic, especially the latter, reduced selenium toxicity in some manner, a second series was set up to determine whether or not lower concentrations of these elements would prevent selenium toxicity and also what effect these elements would have when given at these lower levels in the absence of selenium. Fifty-five rats were divided in groups as shown in table 3.

TABLE 3

GROUP	NUMBER OF RATS	DIET	DRINKING WATER
I	5	Control	Distilled water
II	10	Control	2.5 p.p.m. W in distilled water
III	10	Control	2.5 p.p.m. As in distilled water
IV	10	Seleniferous (11 p.p.m. Se)	Distilled water
V	10	Seleniferous (11 p.p.m. Se)	2.5 p.p.m. W in distilled water
VI	10	Seleniferous (11 p.p.m. Se)	2.5 p.p.m. As in distilled water

The experiment was continued for 75 days. The survivors were then autopsied and their organs examined.

The most important result of this trial was that 2.5 p.p.m. of arsenic in the drinking water would partially, but not completely, prevent liver damage in rats fed a diet containing 11 p.p.m. of selenium in the form of seleniferous wheat. Those rats given arsenic and a seleniferous diet consumed more food than those receiving the seleniferous diet and no arsenic. They also grew more rapidly and were much more active.

The same level of tungsten had no effect in preventing the characteristic symptoms of selenium poisoning. However, all ten rats lived the full length of the experiment while only seven of the ten fed the seleniferous diet and distilled water survived the trial. This reduction of mortality was the only

observed effect of tungsten toward decreasing selenium toxicity.

Neither arsenic nor tungsten at the level of 2.5 p.p.m. in the drinking water of rats receiving a control diet produced any change in food intake, growth, or general appearance from those receiving a similar diet and distilled water.

Water consumption was recorded throughout the trials in order to measure the amount of the added elements which the animals consumed, and also to note the effect of adding these various elements on the volume of water consumed during the entire experimental period. Table 4 shows the average water and element consumption for each group.

As is shown in table 4 there is no significant difference in the water consumption for any of the groups except the group receiving a seleniferous diet with no added element. The water consumption for this group was considerably lower, as is characteristic of the water consumption for selenized animals.

In the other trials in which 5 p.p.m. of arsenic were administered, there was no noticeable difference in the volume of water consumed, as compared with the series which received 2.5 p.p.m. arsenic. However, since the arsenic concentration was twice as great, the total amount of arsenic ingested was also twice as great, and, as has been explained, that amount afforded complete protection against the pathological condition caused by 11 p.p.m. of selenium.

To substantiate the results of the first trial, that 5.0 p.p.m. of arsenic as $NaHAsO_3$ would prevent the toxicity of a diet containing 11.0 p.p.m. of selenium as seleniferous wheat, a third trial was begun. Three groups of two rats each (one male and one female) were used. The first group was fed the control diet and received distilled water. Group 2 received distilled water and the seleniferous diet used in the previous trials. The third group was given the seleniferous diet also but received 5.0 p.p.pm. arsenic as sodium arsenite in its water.

TABLE 4

GROUP	ELEMENT ADDED TO WATER	AVERAGE DAILY WATER INTAKE IN ML. PER RAT PER DAY	AVERAGE DAILY ELEMENT INTAKE IN MG.	AVERAGE TOTAL WATER INTAKE IN LITERS (75 DAYS)	TOTAL ELEMENT INTAKE IN MG. (75 DAYS)
Control diet + distilled water	None	Males—20.6 Females—15.65	None	Males—1.545 Females—1.173	None
Control diet + 2.5 p.p.m. W in water	W	Males—18.7 Females—16.7	Males—0.046 Females—0.041	Males—1.402 Females—1.252	Males—3.50 Females—3.13
Control diet + 2.5 p.p.m. As in water	As	Males—18.3 Females—16.2	Males—0.045 Females—0.0405	Males—1.372 Females—1.215	Males—3.43 Females—3.03
Seleniferous diet + distilled water	None	Males—14.8 Females—12.8	None	Males—1.110 Females—0.960	None
Seleniferous diet + 2.5 p.p.m. W in water	W	Males—12.9 Females—13.8	Males—0.032 Females—0.034	Males—0.967 Females—1.035	Males—2.41 Females—2.58
Seleniferous diet + 2.5 p.p.m. As in water	As	Males—19.0 Females—18.9	Males—0.047 Females—0.047	Males—1.425 Females—1.417	Males—3.562 Females—3.542

The external condition of the rats after being on the experiment for 60 days is shown in figure 1. Those fed the toxic diet and distilled water gained weight very slowly, were inactive, showed loss of hair and reduced food consumption. In contrast, those of the third group which received arsenic in their drinking water and the seleniferous diet showed none

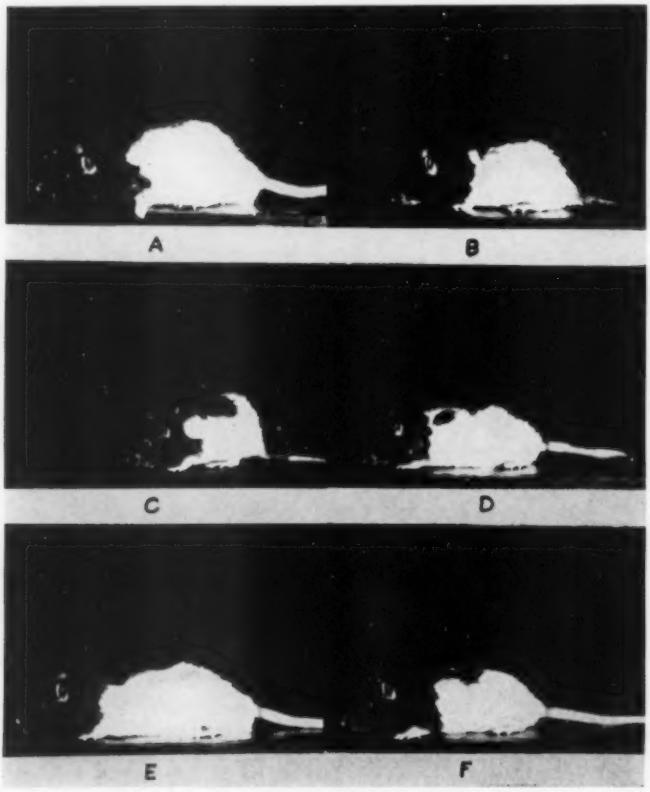

Fig. 1 A, C and E, males; B, D and F, females. A and B, control diet + distilled water; C and D, seleniferous diet (11.0 p.p.m. Se) + distilled water; E and F, seleniferous diet (11.0 p.p.m. Se) + distilled water containing 5 p.p.m. of arsenic as Na_2HAsO_3.

of these symptoms. Their food consumption was normal and their growth paralleled that of the control animals as is shown in figure 2.

Since both of the rats on the seleniferous diet were dead by the sixty-fifth day of the trial the survivors were killed on the seventieth day of the experiment. The condition of their livers at the time of death is shown in figure 3. While

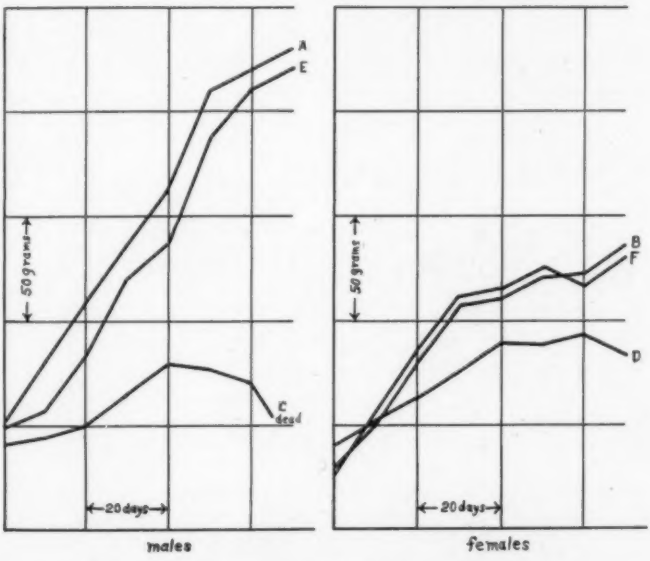

Fig. 2 Growth curves of animals shown in figure 1.

the livers of both rats on the seleniferous diet (C and D) were severely damaged, the livers from the rats getting arsenic and a seleniferous diet (E and F) showed no liver damage at all. This again indicated that arsenic, in some manner, reduced the toxicity of seleniferous wheat when fed to rats. A report of further work on the counteracting effect of arsenic on the toxicity of various forms of selenium is in preparation.

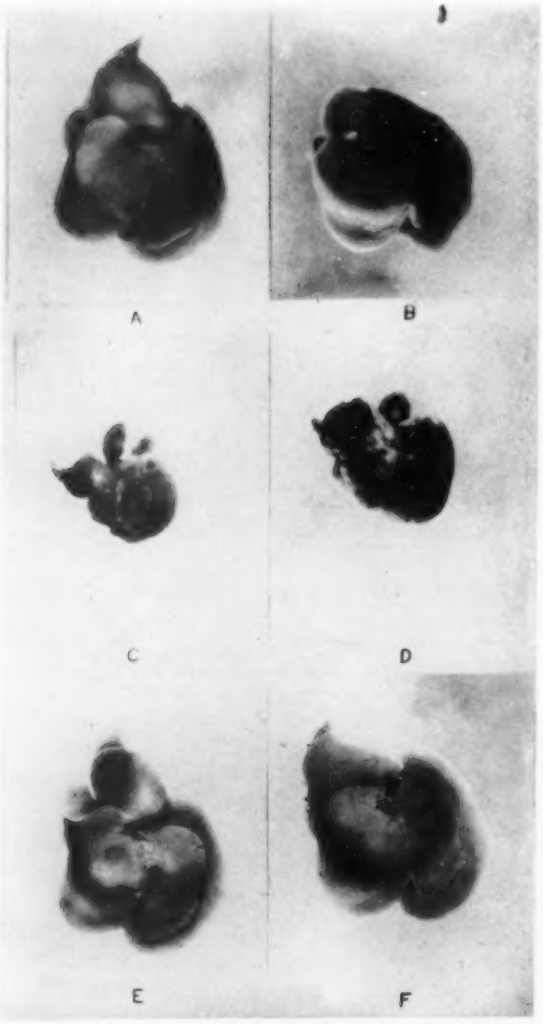

Fig. 3 Livers from corresponding animals in figure 1.

SUMMARY AND CONCLUSIONS

Five p.p.m. of fluorine, molybdenum, chromium, vanadium, cadmium, zinc, cobalt, nickel, and uranium given as water-soluble salts in the drinking water of rats fed a diet containing 11 p.p.m. of selenium from seleniferous wheat caused an increase in mortality.

The same level of tungsten in the form of $Na_2WO_42H_2O$ in the water prevented, to some extent, the typical liver damage caused by the seleniferous diet, and in addition seemed to decrease mortality of rats on such a diet.

Two and one-half p.p.m. of tungsten in the drinking water seemed to reduce the mortality rate of rats fed the seleniferous diet but did not prevent liver damage.

Complete prevention of the symptoms of selenium poisoning was accomplished by giving 5 p.p.m. of arsenic as sodium arsenite in the drinking water.

Two and one-half p.p.m. of arsenic prevented, to some extent, the selenium poisoning symptoms ordinarily caused by a diet containing 11 p.p.m. of selenium from seleniferous wheat. This level of arsenic did not prevent liver damage appreciably, but did bring about a greater food intake and more nearly normal growth.

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THE MINIMUM VITAMIN-A REQUIREMENTS OF NORMAL ADULTS

II. THE UTILIZATION OF CAROTENE AS AFFECTED BY CERTAIN DIETARY FACTORS AND VARIATIONS IN LIGHT EXPOSURE [1]

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ONE FIGURE

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Results reported in the first paper of this series (Booher, Callison, and Hewston, '39) had shown that unit for unit commercial carotene dissolved in cottonseed oil was only about 50 to 60% as efficient in supporting normal dark adaptation in adults as vitamin A in the form of cod liver oil. The ratio of the carotene requirement to the vitamin-A requirement, as determined for five subjects, varied within the rather narrow limits of 1.84 and 1.61.

Wilson, Das Gupta and Ahmad ('37) found that the carotene in raw carrots and in cooked spinach (sak) was about 80 to 90% absorbed by a normal adult when the diet contained an ordinary quantity of fat; when fat was excluded from the diet, however, the absorption of carotene from cooked spinach was reduced to about 50%.

Van Eekelen and Pannevis ('38) reported the carotene in cooked spinach and in cooked carrots to be absorbed only to the extent of 6 and 1%, respectively, when fed to adults in conjunction with a diet of bread, rice, lard, meat, marmite, and 50 mg. of vitamin C. Under similar conditions these workers found that beta carotene dissolved in oil was absorbed to the extent of about 59%.

[1] This project was supported by an appropriation from Bankhead-Jones Funds (Bankhead-Jones Act of June 29, 1935).

Carotene and vitamin-A balance studies are complicated by difficulties in interpretation and by lack of satisfactory methods for recovering these substances from excreta. In order to obtain practical information on the utilization of the carotene in vegetables by human subjects, determinations were made of the physiologic minimum vitamin-A requirements of adults in terms of commercial crystalline carotene and in terms of the carotene as it occurs in peas and spinach. Coincident with these measurements investigations were made on the influence of controlled light exposure and of increased dietary fat, vitamin B_1 and riboflavin in modifying the carotene requirement or utilization of the carotene in vegetables.

EXPERIMENTAL

The details of our method for determining the physiologic minimum vitamin-A requirements of adults and for managing the dietary regimen of the experimental subjects have already been described (Booher, Callison, and Hewston, '39). As in the case of our previous studies the dark adaptation measurements were made with the visual adaptometer described by Hecht and Shlaer ('38).

Chromatographic analyses [2] of the crystalline commercial carotene used in the experiments herein described indicated that it contained 14.0×10^3 mg. and 79.4×10^3 mg. of alpha and beta carotene respectively per 100 gm. Such composition would, presumably, correspond to 1.44×10^6 international units of vitamin A in 100 gm. of the carotene. According to biological assays,[3] using U.S.P. reference cod liver oil as a standard, the carotene contained 1.67×10^6 international units per 100 gm.

Both the peas and the spinach were frozen products purchased at one and the same time in sufficient numbers of 5 pound lots to last throughout the entire investigation. The

[2] We are indebted to L. A. Shinn, Bureau of Dairy Industry, U. S. Department of Agriculture for the chromatographic analyses.

[3] We are indebted to Rosemary Loughlin, for valuable assistance in making the biological assays.

average percentages of dry matter in five samples of the frozen peas and spinach taken from five different lots of each vegetable were 23.5 ± 1.6 and 8.5 ± 0.9%, respectively.

According to chromatographic analyses [4] the peas carried 0.37 mg. and the spinach 5.1 mg. of beta carotene per 100 gm. (raw, fresh weight basis) thus corresponding to 620 and 8500 international units, respectively, of vitamin A per 100 gm. The chromatographic analysis of the carotene in each vegetable was made on a composite sample made up of equal weights of the 5 pound lots of peas and spinach, respectively.

The biological assays [5] for vitamin A-values of the peas and spinach after cooking, using U.S.P. reference cod liver oil as the standard, were 700 and 9500 to 10,000 units, respectively, per 100 gm. (raw basis) of the vegetables. The vitamin-A assays of the vegetables were conducted in parallel with the determinations of the minimum vitamin-A requirements of adults in terms of these same vegetables. The thrice weekly supplements of the green peas and of the spinach fed to the vitamin-A assay rats were taken in successive order from the 5 pound lots of each vegetable.

The vitamin-A values of the cooked peas and cooked spinach are, within the limits of experimental error, in excellent agreement with the vitamin-A values determined by chromatographic analyses.

HUMAN UTILIZATION OF THE CAROTENE IN COOKED PEAS AND SPINACH

The vitamin-A values of the minimum daily intakes of carotene dissolved in cottonseed oil, of cooked peas, and of cooked spinach, which will just support normal dark adaptation in adults, are shown in table 1. In order that comparison of these requirements with those similarly determined for vitamin A in the form of cod liver oil may be facilitated, the results on our five subjects reported earlier are included in this same table. In every case the utilization of the vitamin-A value of the cooked vegetables was superior to that of the

[4] See footnote 2, page 460.
[5] See footnote 3, page 460.

TABLE 1

Summary of minimum vitamin-A requirements of adults in terms of cod liver oil, crystalline carotene, cooked peas, and cooked spinach

SUBJECT	VITAMIN A IN COD LIVER OIL		CAROTENE IN COTTONSEED OIL		RATIO: CAROTENE REQUIREMENT TO VITAMIN-A REQUIREMENT	CAROTENE IN COOKED PEAS		RATIO: CAROTENE REQUIREMENT TO COOKED PEA REQUIREMENT	CAROTENE IN COOKED SPINACH		RATIO: CAROTENE REQUIREMENT TO COOKED SPINACH REQUIREMENT
	Units/kg.	Total units [1]	Units/kg.	Total units [1]		Units/kg.	Total units [1]		Units/kg.	Total units [1]	
H. W.	35.7	1890	57.4	3040	1.61	....	....	....	....	....	....
M. P.	55.8	3900	102.6	7180	1.84	....	....	....	....	....	....
D. E.	52.4	3140	90.0	5400	1.72	....	....	....	....	....	....
H. E.	24.8	1290	42.5	2210	1.71	....	....	....	....	....	....
C. E.	34.3	2060	60.5	3630	1.76	....	....	....	....	....	....
R. E.	....	....	76.3	5180	....	47.0	3200	1.62	....	....	....
B. F.	....	....	96.3	5680	....	57.3	3380	1.68	77.4	4570	1.24
C. C.	....	....	106.2	6795	....	....	....	....	87.0	5570	1.22
C. L.	....	....	....	....	....	....	....	....	101.0	4350	....

[1] The figures in this column include the vitamin-A content of the basal diet plus the vitamin-A value of the supplements.

utilization of the crystalline carotene in cottonseed oil. The ratios of the vitamin-A requirements when fed in the form of commercial crystalline carotene dissolved in cottonseed oil to the requirements supplied in the form of carotene in cooked peas were 1.66 and 1.62 for the two subjects, respectively. When cooked spinach was used in place of cooked peas, these ratios for two subjects were 1.24 and 1.22 respectively. It is apparent, therefore, that the carotene in cooked peas is better utilized than the carotene in cooked spinach. According to the data shown in table 1, it may be concluded that the utilization of the vitamin-A values of cooked peas and cooked spinach is intermediate between the utilization of the vitamin-A value of cod liver oil and that of crystalline carotene in cottonseed oil. Actually the utilization of the vitamin-A value of cooked peas approaches that of cod liver oil while the utilization of the vitamin-A value of cooked spinach is more nearly like that of crystalline carotene dissolved in cottonseed oil.

The average composition of the experimental diets for three of the subjects, for whom the minimum physiologic requirements for vitamin A in terms of cooked peas and of cooked spinach were determined, is summarized in table 2. The data shown graphically in figure 1 represent the general type of dark adaptation response encountered in measurements of the minimum physiologic requirements for vitamin A in terms of a specific food item, in this case cooked green peas.

UTILIZATION OF THE VITAMIN-A VALUE OF CAROTENE AS INFLUENCED BY CERTAIN DIETARY FACTORS AND BY VARIATIONS IN LIGHT EXPOSURE

From time to time various dietary factors have been mentioned as potential promoters of carotene or vitamin-A utilization. Increased intake of fat or of thiamin are the most frequently mentioned of these dietary factors suggested as promoting the utilization of carotene.

The observation of Aykroyd ('30) to the effect that fishermen in Labrador conserve their ability to see in dim light by keeping one eye shielded from light during the day suggests

TABLE 2

Average composition of experimental diets of three subjects for whom the minimum physiological requirements for vitamin A in terms of cooked peas and of cooked spinach were determined. Quantities refer to daily intakes

DESCRIPTION OF EXPERIMENTAL PERIODS	TOTAL CALORIES	PROTEIN	FAT	MINERALS			VITAMINS			
				Ca	P	Fe	A	Thiamin	Ascorbic acid	Riboflavin
Subject B. F.		*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*I.U.*[1]	*I.U.*	*I.U*	*S.U.*[2]
1. Depletion period	1840	77	58	1.0	1.3	0.026	80	480	1830	610
2. Vitamin-A deficient diet + carotene in oil	1970	71	64	1.2	1.4	0.024	5680	450	1730	590
3. Depletion period	1760	74	57	1.1	1.4	0.023	70	450	2140	600
4. Vitamin-A deficient diet + cooked peas[3]	1910	87	48	1.2	1.7	0.027	3380	800	2930	710
5. Vitamin-A deficient diet + cooked spinach	1700	77	49	1.1	1.4	0.024	4570	400	2340	580
Subject R. E.										
1. Depletion period	2380	90	83	1.2	1.5	0.026	80	550	2020	650
2. Vitamin-A deficient diet + carotene in oil	2400	115	88	1.2	1.8	0.030	5180	600	2000	670
3. Depletion period	2390	96	71	1.0	1.4	0.028	70	630	2000	600
4. Vitamin-A deficient diet + cooked peas	2530	108	81	1.0	1.9	0.031	3200	1000	2530	710
Subject C. C.										
1. Depletion period	1780	71	52	1.1	1.3	0.024	80	440	2210	590
2. Vitamin-A deficient diet + carotene in oil	1760	73	46	1.1	1.4	0.023	6795	480	2240	600
3. Depletion period	1760	76	30	1.2	1.4	0.024	75	500	2420	580
4. Vitamin-A deficient diet + cooked spinach	1650	72	44	1.1	1.3	0.025	5570	460	3580	610

[1] International units.

[2] Sherman-Bourquin units.

[3] A short period on the vitamin-A deficient diet alone was interposed between periods 4 and 5 during which the composition of the diet was very similar to those given for periods 1 and 3.

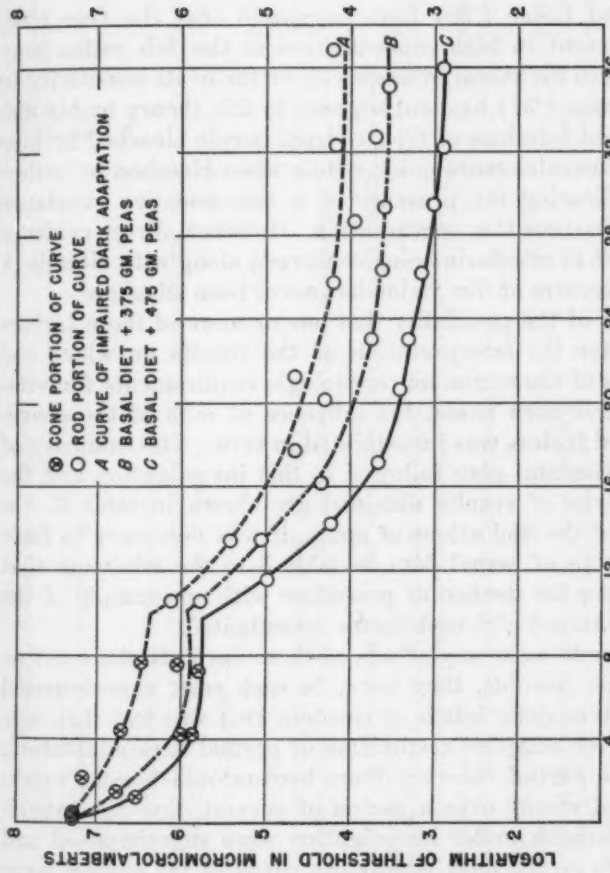
LOGARITHM OF THRESHOLD IN MICROMICROLAMBERTS
TIME IN MINUTES
⊗ CONE PORTION OF CURVE
○ ROD PORTION OF CURVE
A CURVE OF IMPAIRED DARK ADAPTATION
B BASAL DIET + 375 GM. PEAS
C BASAL DIET + 475 GM. PEAS
A
B
C

Fig. 1 Dark adaptation curves for subject B. F., showing recovery of normal dark adaptation with addition of cooked peas to the diet.

the possibility of light exposure having an appreciable influence on vitamin-A requirements.

The presence of riboflavin in the retinas of cattle, sheep, dogs, chickens and fish was reported by Euler and Adler ('34). Adler and Euler ('38) have suggested that the free riboflavin present in high concentration in the fish retina may take part in the visual processes by virtue of its sensitivity to light. Chase ('37) has lent support to this theory by his discovery that solutions of frog's visual purple bleached by blue light regenerates more quickly than when bleached by yellow light, indicating the presence of a blue-sensitive substance which influences this regeneration. However, direct evidence in support of riboflavin being concerned along with vitamin A in the processes of dim vision has never been advanced.

In view of the possibility that one or more of these factors might alter the interpretation of the results on which our estimates of the minimum physiologic requirements for vitamin A have been based, the influence of each of the above-mentioned factors was investigated in turn. The summary of the experimental plan followed in this investigation and the general type of results obtained are shown in table 3. On account of the limitations of space, it was necessary to limit presentation of actual data in table 3 to the minimum that would show the method of procedure with an example of the results obtained with each factor investigated.

In order to make any effects of these variants stand out as sharply as possible, they were, in each case, superimposed upon a given daily intake of carotene that was less than was required for complete restoration of normal dark adaptation. When the partial recovery from hemeralopia became established and steady over a period of several days the experimental variants under investigation were superimposed and the effects on the dark adaptation curve of the subject were determined over another period of several days. Under these conditions any improvement in the dark adaptation response would indicate better utilization of the carotene as a result of the superimposed experimental variant.

TABLE 3

Data showing the influence of variations in intake of thiamin, riboflavin and dietary fat and of light exposure on vitamin A (supplied as carotene) requirements of adults

SUBJECT AND EXPERIMENTAL CONDITIONS [1]	DURATION OF EXPERIMENTAL PERIOD	AVERAGE LIGHT THRESHOLDS [2]	
		After 6 minutes dark adaptation	After 30 minutes dark adaptation
B. F. (female) age 32; height 164 cm.; weight 59 kg.:	*days*	*log μμ lamberts*	*log μμ lamberts*
Before depletion	....	5.67 ± 0.21	2.96 ± 0.08
Vitamin-A deficient diet	48	6.11	4.00
Vitamin-A deficient diet + carotene (3300 units vitamin A)	4	5.77 ± 0.06	3.33 ± 0.02
Vitamin-A deficient diet + carotene (3300 units vitamin A) + 170 μ grams thiamin	14	6.02 ± 0.06	3.51 ± 0.10
Vitamin-A deficient diet + carotene (5605 units vitamin A)	4	5.70 ± 0.05	3.03 ± 0.09
C. L. (female) age 19; height 155 cm.; weight 43 kg.:			
Before depletion	....	5.72 ± 0.15	3.17 ± 0.12
Vitamin-A deficient diet	22	6.20	4.20
Vitamin-A deficient diet + cooked spinach (1900 units vitamin A)	14	5.95 ± 0.19	3.69 ± 0.66
Vitamin-A deficient diet + cooked spinach (1900 units vitamin A) and wearing goggles in daytime	3	5.95 ± 0.06	3.48 ± 0.07
Vitamin-A deficient diet + cooked spinach (1900 units vitamin A) + 200 μ grams thiamin + 300 μ grams riboflavin	4	6.00 ± 0.00	3.46 ± 0.08
Vitamin-A deficient diet + cooked spinach (3325 units vitamin A)	7	5.94 ± 0.07	3.33 ± 0.05
Vitamin-A deficient diet + cooked spinach (3325 units vitamin A) and with 6 hours daily exposure to bright sunshine	5	5.92 ± 0.08	3.29 ± 0.10
Vitamin-A deficient diet + cooked spinach (4275 units vitamin A)	8	5.76 ± 0.04	3.02 ± 0.02
Y. E. (male) age 19; height 178 cm.; weight 67 kg.:			
Before depletion	....	5.86 ± 0.18	3.25 ± 0.18
Vitamin-A deficient diet (35% of total day's calories from fat)	40	6.15	3.70
Vitamin-A deficient diet + carotene (4355 units vitamin A)	16	5.83 ± 0.10	3.31 ± 0.13
Vitavin-A deficient diet + carotene (4355 units vitamin A) with proportion of calories from fat increased to 40%	15	6.18 ± 0.17	3.50 ± 0.00
Vitamin-A deficient diet + carotene (5025 units vitamin A)	21	5.90 ± 0.08	3.16 ± 0.18

[1] All dietary supplements mentioned are per diem.

[2] Thresholds were measured at 7° on nasal side of fovea, image a 3° test spot. The values for subjects before depletion are averages of 9 to 21 measurements taken on different days. The second set of threshold values for each subject is for the last day of the depletion period. The remaining thresholds are representative averages of determinations made toward the latter part of the periods described; the measurements, in each case, were made 18 to 22 hours subsequent to the administration of the previous day's vitamin supplements.

According to the results shown in table 3 it would appear that a daily intake of thiamin in excess of 400 to 600 international units (see table 2 for thiamin values of the basal vitamin A-deficient diets) did not improve the utilization of carotene. In addition to the results shown for subject B. F., the findings were confirmed by results obtained with a male subject with whom the same experimental plan was carried out. The dark adaptation response of these two adults already receiving daily around 60% of the physiologic minimum requirement of carotene necessary for the support of normal dark adaptation was not improved as a result of increased intakes of thiamin. Neither did enrichment of the diet in thiamin and riboflavin together show any improvement in the average light threshold values (see data on subject C. L., table 3) when superimposed upon a supplement of cooked spinach that was inadequate to support fully normal dark adaptation. The vitamin A-deficient basal diet of this subject previous to enrichment with the 300 micrograms of pure riboflavin carried about 600 Sherman-Bourquin units of vitamin G (probably equivalent to around 1800 to 2400 micrograms of riboflavin) per day. The cooked spinach supplements used as a source of vitamin A were consumed by the subject in equal portions at the noon and evening meals.

The average fat content of the vitamin A-deficient diets of the experimental human subjects was such that approximately 30 to 35% of the total calories were provided in the form of fat. This level of dietary fat corresponds to that ordinarily found in average American dietaries. As shown in table 3 (see data on subject Y. E. as the example) increase in fat intake beyond this level had no appreciable effect upon the utilization of carotene. This observation was confirmed by results similarly obtained for a young adult female subject.

One subject (see data for subject C. L., in table 3) submitted for a 3-day period to wearing closely fitted dark goggles of the type frequently used to protect the eyes from the intense light of high voltage welding-arcs. The transmission of the darkened glass was approximately 2%. The goggles

were worn steadily with the exception of about 1 hour a day during the entire waking period of the 3-day interval. Preliminary to each measurement of dark adaptation during the 3-day period this subject was adjusted gradually to ordinary indoor daylight before proceeding with the visual measurements. It was not feasible for us to continue this variation in experimental conditions beyond this one 3-day interval and we have not, to date, had the opportunity to repeat the experiment with other subjects. However, from the observation of this one case it would appear that if any lowering of the vitamin A requirement attended the diminution of exposure of the eyes to ordinary daylight, its magnitude is so small as to be of no practical significance in defining minimum vitamin A requirements.

After a 7-day period of increased but inadequate intake of carotene in the form of spinach, this same subject was exposed to bright outdoor sunshine for 6 hours daily over a period of 4 days. This exposure to light was without appreciable effect on the threshold values measured during the course of dark adaptation. These results are in accord with the fact that maintenance of a normal visual purple mechanism represents only a very small part of the physiologic need of the body for vitamin A.

SUMMARY

The vitamin A assay values of cooked peas and cooked spinach, as determined by the rat-growth method using U.S.P. reference cod liver oil as the vitamin A standard, were found to be in excellent agreement with the carotene analysis of spinach as determined by chromatographic analysis.

A daily intake of approximately 47 and 57 units of vitamin A, respectively, per kilogram of body weight was necessary for the maintenance of normal dark adaptation in two normal adults when the vitamin A value of the diet was derived almost entirely from the carotene in cooked green peas.

A daily intake of approximately 77, 87 and 101 units of vitamin A, respectively, per kilogram of body weight was

required for the same response in three adults when the vitamin A value of the diet was derived almost entirely from the carotene in cooked spinach.

For maintenance of normal dark adaptation in adults the utilization of the vitamin A values in cooked peas and cooked spinach is intermediate between those for cod liver oil and for commercial crystalline carotene dissolved in cottonseed oil. The utilization of the vitamin A value of cooked peas was better than that of cooked spinach.

A daily intake of thiamin in excess of 400 to 600 international units does not appear to improve the utilization of carotene by adults nor to diminish their minimum physiologic requirement for vitamin A.

A daily intake of riboflavin in excess of about 1800 to 2400 micrograms was not accompanied by increased utilization of carotene nor by diminution of the minimum physiologic requirement for vitamin A in adults.

Dietary fat in an ordinary mixed diet in excess of that which provides about 30 to 35% of the total caloric intake showed no beneficial effects upon the utilization of carotene by normal adults.

A marked increase or a marked decrease in exposure of the eyes of normal adults to ordinary light sources is probably not accompanied by significantly altered requirements for vitamin A.

ACKNOWLEDGMENTS

We wish particularly to acknowledge our indebtedness to Edna Mohagan for her invaluable contribution in planning and preparing the meals for the experimental subjects and in making the daily calculations of food intakes; to Pearl Anderson and Virginia Knowles who assisted with the dark adaptation tests; and to those of our associates in the bureau of home economics who served as subjects for these experiments.

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BASAL METABOLISM OF THE ADULT RABBIT AND PREREQUISITES FOR ITS MEASUREMENT

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ONE FIGURE

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The unusually large range in the basal heat production of the rabbit, as reported by previous investigators who give heat values differing by more than 100%, has challenged us to investigate this subject further. As already pointed out (Smuts, '35; Friedgood and Bevin, '39), the conditions prerequisite for basal metabolism measurements on the rabbit have not been well defined; the statements concerning these conditions are conflicting, and no comprehensive study of the basal metabolism of this animal has been made. Because adult rabbits have a sevenfold range in weight and yet are of comparable proportions, they offer ideal conditions to study the specific effect of size upon metabolism within the same species. Hence this investigation was undertaken with three objectives in view, 1) to determine the conditions necessary for basal metabolism measurements on the rabbit, 2) to establish this animal's basal metabolic level, and 3) to study the influence of size on heat production within the same species. The first two of these objectives will be discussed in this report. The third will be considered in a subsequent paper.

TECHNIC

The metabolism was measured with two forms of respiration apparatus, both on the open-circuit principle. For 3- to

7-kg. rabbits, the volume of ventilating air was measured by dry gas meters recording to 0.1 liter, and for 1- to 3-kg. rabbits, by a wet gas meter recording to 0.01 liter. Aliquot samples were collected in 1-liter spirometers for the small rabbits and in the Fox bag (Carpenter and Fox, '31; Benedict, '36) for the large rabbits. The samples of ventilating air were analyzed on the apparatus of Carpenter ('33). The size of chamber varied according to the size of the animal, its capacity ranging from 5.0 liters for a 1-kg. rabbit to 16 liters for a 7-kg. rabbit. Constancy (usually within 1%) in the ventilation rate from period to period was maintained by means of a Murrill (1898) regulator.

The apparatus were frequently tested for tightness. In alcohol control tests the respiratory quotients ranged from 0.644 to 0.669 in the individual periods of measurement. The average ratio in seven experiments with the larger apparatus was 0.660 and in four experiments with the smaller apparatus, 0.659. The gas-analysis apparatus was checked on nearly every experimental day by analyses of outdoor air. These analyses (119) showed a range in carbon dioxide of from 0.029% to 0.033% (average 0.030%), and in oxygen of from 20.934% to 20.948% (average 20.939%), with standard deviations of ± 0.001 and ± 0.003, respectively.

The chamber temperature (indicated by a mercurial thermometer, with its bulb in the outgoing air pipe at its exit from the chamber) was held at the desired level by placing the chamber in a larger, water-jacketed chamber, which provided several inches of air space between the water jacket and the metabolism chamber. Thermostatic control was frequently used to maintain the temperature of the animals' room within the desired limits, and the temperature was recorded by a calibrated thermograph. Body temperature was measured by thermocouples (Lee, '39).

In all experiments graphic records of activity were obtained by placing the chamber on a platform, suspended by a pneumograph.

PREREQUISITES FOR BASAL METABOLISM MEASUREMENTS

The conditions necessary for basal metabolism measurements on any animal species have been pointed out independently by Benedict ('38) and Giaja ('38). Both emphasize absence of muscular activity, post-absorptive condition, zone of thermic neutrality, adaptation to this zone, normal nutritive state, the animal's age (avoidance of the complicating factors of growth and senility), and time of day at which the measurement is made. Le Breton ('34 a) has partially set forth the conditions for determination of the rabbit's basal metabolism. The conflicting data and statements of several careful investigators, however, concerning the zone of thermic neutrality and the time when the post-absorptive condition is reached leave uncertainty as to these factors.

Animals used. Seventy-five rabbits were obtained from three sources. One group was obtained from local breeders. Two other groups were lent to us, one through the kindness of Prof. William E. Castle of the Bussey Institution of Harvard University, and the other through the kindness of Prof. Gregory Pincus of the Department of Physiology of Harvard University. Rabbits 10, 40, 47, 52, and 62 were 7 to 9 months old; rabbit 36 was 5 years old, and the others ranged in age from 10 months to 4 years at the time of their first metabolism measurements. The average age was 1¾ years, and the maximum age in any experiment, 6 years. As the rabbit's life span may be at least 10 years (Tegge, '36), and as rabbits mature sexually at about 6 months (Fangauf and Immenkamp, '38), our rabbits were all adult but in no sense senile. They were also in good nutritive state, as indicated by their general appearance, by dissection of a number of animals, and by comparison of their body weights with standard weights for respective breeds. Their weights ranged from 1 to 7 kg. They were housed in individual cages and, unless fasting for experiments, were fed each morning Purina rabbit chow, supplemented frequently by carrots, carrot tops, and parsley.

Activity. As excessive activity can double the rabbit's metabolism, this factor has been eliminated in the following

discussions of results in two ways. Only measurements in periods when no appreciable activity occurred have been considered, and of these, only period values within 10% of the minimum value found in any experiment were included in the average for the experiment. Usually each average value represents from three to five periods of measurement, occasionally only two periods or all six periods.

Post-absorptive state. The two criteria for establishing absence of digestive activity are the respiratory quotient and the heat production. An analysis of the respiratory quotients (measured at approximately 28°C.) according to the length of time food had been withheld from the rabbits reveals that a 24-hour fast did not always result in quotients of fat. The 165 values at the twenty-fourth hour after food ranged from 0.67 to 0.86, but the greatest number were 0.74, which was the average value. At the forty-eighth hour the nineteen quotients ranged from 0.67 to 0.74 and averaged 0.71. The fifteen quotients at the seventy-second hour also averaged 0.71, and the range was from 0.68 to 0.73. In eleven experiments (also at about 28°C.), in which the exact time of the last food ingestion was known, the maximum quotients within 6 hours of this time varied from 0.80 to 0.96, over half of them being 0.90 or above. The ranges and averages at the twenty-fourth hour (eleven experiments), forty-eighth hour (nine experiments), and seventy-second hour (five experiments) of fasting were as follows:

Hours fasting	*Range*	*Average*
24	0.70 to 0.79	0.74
48	0.68 to 0.75	0.71
72	0.68 to 0.73	0.70

Comparison of the heat production and the simultaneously determined respiratory quotient at various stages of fasting is given in table 1 for sixteen experiments, most of them at about 28°C. The heat production had only a slight tendency to decrease as the fast progressed and, on the whole, was essentially the same whether the rabbit had been fasting 24, 48 or 72 hours. Exceptions are noted in experiments e, g, i and

m, in which the heat production decreased between the twenty-fourth and forty-eighth hours, although quotients at the fat level were found at the twenty-fourth hour. After 24 hours of fasting there was no consistent tendency for a decreasing respiratory quotient to be accompanied by a lowered heat production. In experiments a, c, f and k the respiratory quotient decreased as fasting continued from the twenty-

TABLE 1

Relation of heat production per square meter of body surface[1] per 24 hours and respiratory quotient to length of fast

EXPERIMENT[2]	RABBIT NO.	WEIGHT	HOURS FASTING							
			1 to 6 hours		24 hours		48 hours		72 hours[3]	
			R. Q.	Calories	R. Q.	Calories	R. Q.	Calories	R. Q.	Calories
		kg.								
a	9	1.39	0.80	807	0.76	602	0.72	724		
b	16	4.00	0.92	892	0.77	821	0.70	[4]	0.73	812
c	55	4.00	0.91	1026	0.77	753	0.72	748		
d	2	1.14			0.68	522	0.72	528	0.70	499
e	2	1.16	0.78	723	0.70	605	0.72	477	0.72	503
f	2	1.20			0.74	518	0.70	520	0.70	499
g	9	1.44	0.80	648	0.71	580	0.67	557	0.70	544
h	9	1.35			0.72	553	0.71	562	0.71	541
i	9	1.34			0.70	624	0.72	580	0.70	547
j	12	1.61			0.72	601	0.71	589	0.70	623
k	65	3.55			0.76	632	0.73	676	0.69	623
l	47	3.76			0.72	652	0.71	642	0.70	617
m	63	4.77	0.79	857	0.70	856	0.70	829	0.68	923
n	63	4.56			0.73	858	0.69	885	0.72	817
o	63	4.28			0.73	810	0.70	829	0.71	781
p	68	5.15			0.74	551	0.70	543	0.69	560
Average[5]					0.72	643	0.71	632	0.70	621

[1] Calculated from formula $S = 0.001w\left(\frac{K \times w^{2/3}}{10{,}000}\right)$ in which S represents square meters; w, weight in grams, and K = 10.

[2] The rabbits were kept at essentially the experimental temperature for 24 hours prior to the first measurements and also between measurements; all experiments were at 27° to 32°C. except experiments a, b, c and k which were at 24°C.

[3] Observations at the ninety-sixth hour gave the following results: experiment i, R. Q. = 0.69, 567 cals.; experiment j, R. Q. = 0.69, 604 cals.; experiment o, R. Q. = 0.70, 727 cals.

[4] Active.

[5] Not including experiments a, b and c.

fourth to the forty-eighth hour, but the level of the heat production was not lowered. There were also experiments (a and k) when the minimum or approximately the minimum heat production occurred with the highest respiratory quotient. Thus the minimum heat production is not dependent on or insured by a respiratory quotient of fat. In table 1 the average heat production at the seventy-second hour was only 3% below that at the twenty-fourth hour. At the ninety-sixth hour the heat production in two of three experiments was the same as at the seventy-second hour. Hence, between the twenty-fourth and the seventy-second hours of fasting, any effect of absorption of food on the metabolism can be considered negligible and the heat production can be considered to be at a level, even though the respiratory quotient may not be that of fat.

A large number of experiments revealed that the respiratory quotient does not change significantly in the course of 2 to 3 hours, when the rabbit has been fasting at 27° to 28°C. for 22 to 26 hours. In representative experiments, each of six 25- to 30-minute periods, the following quotients were noted:

Rabbit no.	*Period no.*					
	1	2	3	4	5	6
3	0.73	0.72	0.72	0.73	0.73	0.72
23	0.71	0.70	0.72	0.70	0.72	0.71
24	0.71	0.71	0.72	0.72	0.73	0.73
42	0.73	0.75	0.74	0.74	0.76	0.73

As the quotients did not vary significantly in any one experiment, it is concluded that the average of any two individual quotients in a given experiment will be representative of all the quotients. Therefore the respiratory quotient was determined usually in only two (non-consecutive) periods of the six periods of each basal experiment, and the two quotients were averaged. In the other periods only the carbon-dioxide production was measured. The heat production in the different periods was then calculated by applying the caloric value of carbon dioxide for this average respiratory quotient

to the measured carbon-dioxide production, reduced to standard conditions of temperature and pressure.

Zone of thermic neutrality and period of habituation to temperature. In figure 1, curves are given showing the course of the heat production per square meter of surface area of rabbit 12 as the environmental temperature was increased. The animal had been fasting 24 hours at the start of each experiment and remained at each temperature level for about

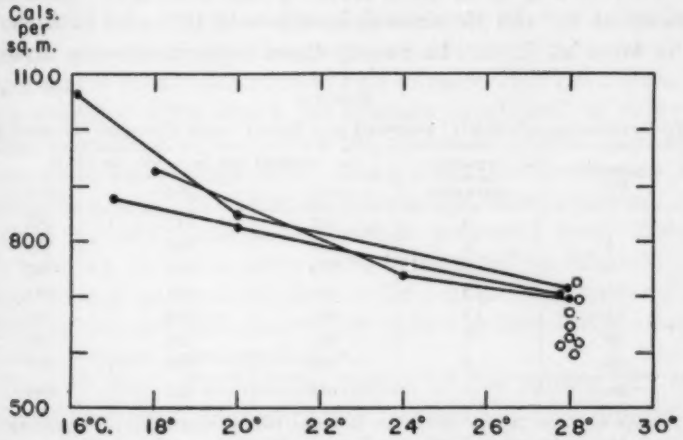

Fig. 1 Influence of environmental temperature upon heat production per square meter of surface area per 24 hours—rabbit 12. The animal had been 24 hours without food at the start of each experiment. Each curve represents the results obtained at successively higher environmental temperatures on the same day, after the rabbit had been living at 17° to 21°C. for more than 3 weeks. The measurements at each temperature did not start until the rabbit had been at the given temperature for about 40 minutes or longer. The hollow circles represent measurements made at 28° after the rabbit had been living 24 hours or more at this temperature. Each plotted value represents the average of several periods of measurement.

2 hours (including about 40 minutes prior to the metabolism measurement). Although these particular curves are based upon the results obtained with one rabbit, they are also representative of those found with seven other rabbits. The metabolism was lowered by the increased temperatures. This

is contrary to the findings of several investigators (Bacq, '29 a, '29 b; Le Breton and Schaeffer, '33; Le Breton, '34 a), who reported thermic neutrality for the rabbit to be as low as 20°C. Measurements at 28°, 30°, and 32°C. on the same day are recorded in table 2. The rabbits had been fasting 20 to 72 hours and had been living at 26° to 33°C. Although there were variations, in general the heat production was essentially constant, the average values at the three temperatures differing from each other by less than 1%. Measurements at 34° and 36° showed increases of 16% and 15% above the level at 32°C. In twenty-three experiments on sixteen

TABLE 2

Heat production of rabbits[1] *measured at different temperatures on the same day*

RABBIT NO.	NUMBER OF EXPERIMENTS	CALORIES PER SQ.M. PER 24 HOURS		
		28°C.	30°C.	32°C.
2	3	542	512	525
4	1	617	586	616
9	3	558	563	562
12	1	649	615	584
13	1	673	669	642
19	1	682	694	702
63	2	851	930	906
Av.		653	653	648

[1] Kept at from 26° to 33°C. for 24 hours before experiment; post-absorptive.

rabbits habituated for 24 hours to 28° and measured both at 28° and at 24° on the same day, the metabolism at 24° averaged 5% higher than that at 28°C.; a few measurements at 26° were at an intermediate level. With three of the sixteen animals the heat values were lower at 24° than at 28°, averaging 3.8% lower. From the data presented, as well as supplementary data, it is concluded that the zone of thermic neutrality for the rabbit is from 28° to 32°C.

With one exception, the open circles in figure 1 at 28° are below the levels of the curves at this temperature. For 24 hours prior to the measurements represented by these open circles the rabbit had been living at 28°, whereas previous to the measurements at the lower temperatures (represented by

the curves) it had been at about 18°C. From the data on a number of rabbits studied under varying conditions as to environmental temperature, we find that if rabbits have been living below thermic neutrality but above 20°C., habituation to thermic neutrality for an additional 24-hour period prior to measurement will permit normal adjustment of the metabolism to the basal level.

Adjustment to laboratory manipulations. As noted by Le Breton ('34 a) and others, the first experiment made on an animal is often significantly high because of the animal's lack of adjustment to laboratory procedures. In the initial experiments on animals studied on 4 or more days the metabolism averaged 6.3% above the average basal level in subsequent experiments; in half of the instances it was 7.5% or more above the average level. The initial experiment on each animal has been omitted from its average value reported in table 3 (p. 484), except for rabbits borrowed from other laboratories, as these latter were accustomed to laboratory handling and as consideration of their initial measurements shows agreement with the average values obtained with other rabbits in numerous experiments.

Time of day. The heat production of five rabbits (3.5 to 4.6 kg.) at thermic neutrality was measured at four different times of day. The animals had been 24 hours without food at the start of each experiment. Although the length of the fast was greater as the experiment progressed, any effect of this factor in obscuring the analysis with regard to time of day was avoided by varying the time of starting the experiments. The results are as follows, the values in italics indicating the initial measurements.

Rabbit no.	*Calories per sq. m. per 24 hours*			
	9 A.M.	2 P.M.	9 P.M.	2 A.M.
41	*840*	752	756	791
53	683	*716*	730	728
58	*578*	572	592	556
59	783	*682*	739	711
64	656	648	*715*	673
Average	708	674	706	692

There is some variation between the measurements on any one animal but no consistent variation ascribable to time of day. Le Breton ('34 b) also failed to find any consistent diurnal variation in the rabbit's metabolism. Similarly, no characteristic daily rhythm was found in the rabbit's body temperature (Lee, '39).

BASAL METABOLISM

Variability in the individual rabbit's metabolism from day to day. Basal metabolism measurements under the conditions outlined above were made on seventy-four rabbits, of which thirty-four were studied in three or more experiments. Even under presumably uniform conditions, some variability existed between measurements repeated from day to day on the same rabbit. Among eight rabbits studied on 8 or more days each, the mean maximum variation in heat production per square meter of surface area from the average heat production was $\pm 15.3\%$. The standard deviation of the percentage differences between the individual values for each animal and its average value per square meter was $\pm 7.8\%$ in the ninety-six measurements on these eight animals. For the thirty-four animals measured on 3 or more days the standard deviation of the percentage differences was $\pm 6.9\%$.

Average basal metabolism of the rabbit species. The average results for our seventy-four rabbits are summarized in table 3. The heat production is expressed in total calories per 24 hours and in calories per square meter of surface area ($S = 0.001w^{2/3}$; w = weight in grams). The metabolism increases with size, the total heat production ranging from 60 calories for a 1.2 kg. rabbit to 335 calories for a 7-kg. rabbit, and the heat production per square meter from 534 to 914 calories. No single value on any of the common bases may be given to represent the heat production of the rabbit species, but the data in table 3 may be averaged according to weight as follows:

Weight group kg.	Number in group	Average weight kg.	Calories per 24 hours Total	Per sq.m.
1	20	1.52	82.6	624
2	13	2.46	119.3	654
3	19	3.57	163.7	699
4	14	4.33	191.1	721
5	7	5.33	232.7	761

A number of investigators have reported heat values for rabbits expressed with reference to surface area but have failed to state the formula used for calculating the area, which prevents intelligent comparison of the results. We here enter a plea for the use of a uniform method of making such calculations. In those instances where comparisons are possible, we find that most of the previously published values (calculated per $0.001w^{2/3}$; w = weight in grams) for the basal heat production of the rabbit are higher than ours. Possibly the explanation is that the measurements were not made under all the prerequisite conditions outlined above. Our values are among the minimum. Other values that agree closely with ours, when weight is taken into consideration, are those reported by Laufberger ('25), Ghoneim ('30), Le Breton and Schaeffer ('33), Le Breton ('34 b), and Smuts ('35), and some of the minimum values of Webster, Clawson and Chesney ('28).

Sex. Comparison of the data with regard to sex shows essentially the same range in percentage variability from the average trend for total heat production referred to weight for each sex, but the twenty-three males have a metabolism 2% higher than that of the females when their average metabolic trends with increasing weights are considered. Hence, a sex difference, which has been found pronouncedly in humans (Gephart and DuBois, '16; Harris and Benedict, '19) and to a less extent in rats (Horst et al., '34 a, '34 b), is suggested by these data on rabbits.

Rectal temperature in relation to metabolism. The normal limits of the rectal temperatures of adult rabbits are from 38.4° to 41.1°C. (Lee, '39). The rectal temperature measure-

TABLE 3

Basal metabolism of rabbits[1]

(*Average values*)

RABBIT NO., BREED,[2] AND SEX			WEIGHT	NUMBER OF EXPERIMENTS	RECTAL TEMP.[3]	R. Q.	HEAT PRODUCED PER 24 HOURS	
							Total	Per sq.m.[4]
			kg.		°*C.*		*Cals.*	*Cals.*
1	P	♂	1.15	3	39.1	0.72	77.7	706
2	P	♂	1.18	14	39.3	0.72	59.8	534
3		♂	1.18	1		0.73	69.6	621
4		♀	1.22	2		0.73	67.8	592
5	P	♀	1.28	5	38.7	0.72	72.3	613
6		♂	1.29	1		0.75	75.2	632
7		♂	1.31	2		0.73	66.5	558
8	P	♂	1.31	5	39.1	0.70	75.5	629
9	P	♀	1.37	21	39.4	0.72	72.7	591
10		♂	1.43	2		0.73	69.5	550
11			1.49	1	39.3	0.71	78.2	602
12	P	♀	1.52	17	39.6	0.71	82.1	622
13		♀	1.70	2	39.8	0.75	92.2	649
14	E	♂	1.70	1		0.76	91.4	644
15		♀	1.78	1		0.73	83.8	570
16	D	♂	1.81	1		0.72	97.8	656
17		♀	1.86	2	39.2	0.74	86.7	574
18		♀	1.90	2	39.6	0.83	108.0	706
19		♀	1.93	2		0.74	105.9	684
20		♀	1.97	1		0.74	118.3	754
21		♀	2.06	1	39.4	0.71	95.4	589
22	H	♀	2.06	2	38.6	0.71	103.0	638
23		♂	2.17	1	38.6	0.71	101.2	602
24	H	♂	2.23	3	39.0	0.72	91.4	535
25		♂	2.38	1		0.77	140.3	788
26		♀	2.40	1		0.76	112.8	630
27	D	♂	2.42	4	39.0	0.73	128.4	713
28		♀	2.47	1	39.5	0.76	116.1	634
29		♀	2.67	1	39.4	0.78	127.8	666
30		♀	2.69	1	39.2	0.75	112.1	581
31	C	♀	2.79	4	39.8	0.74	143.1	723
32		♀	2.80	1	39.1	0.74	138.4	696
33	J	♂	2.84	1		0.74	141.4	703
34	F	♂	3.11	3	39.6	0.73	118.1	554
35		♀	3.13	1	39.7	0.77	140.8	658
36		♂	3.20	1		0.74	157.8	727
37	Z	♀	3.21	4	39.6	0.74	132.5	608
38	Z	♀	3.30	3	39.2	0.73	149.1	672
39		♀	3.37	1	39.4	0.74	182.8	812
40	Z	♂	3.43	1	39.6	0.74	196.8	867

TABLE 3 (*Continued*)

RABBIT NO., BREED[2] AND SEX			WEIGHT	NUMBER OF EXPERIMENTS	RECTAL TEMP.[3]	R. Q.	HEAT PRODUCED PER 24 HOURS	
							Total	Per sq.m.[4]
			kg.		°*C.*		*Cals.*	*Cals.*
41	Z	♀	3.55	7	39.7	0.76	161.1	691
42	E	♀	3.56	1	39.1	0.74	141.2	606
43		♀	3.57	1	39.1	0.74	121.1	518
44	Z	♀	3.69	4	40.3	0.76	184.8	773
45		♀	3.69	2	39.4	0.74	164.8	691
46		♀	3.77	1		0.76	218.4	902
47	Z	♀	3.81	3	39.6	0.71	160.3	657
48		♀	3.83	1	39.0	0.75	170.8	697
49		♀	3.87	2	39.8	0.73	180.4	734
50	E	♀	3.89	2	39.2	0.74	148.7	601
51	Z	♂	3.97	1		0.71	159.4	635
52	Z	♂	3.97	3	40.2	0.73	221.7	883
53	Z	♀	4.02	8	40.0	0.76	189.6	750
54	Z	♀	4.03	5	39.8	0.74	187.8	742
55	Z	♀	4.04	3	39.8	0.74	182.1	717
56	Z	♀	4.11	4	39.7	0.74	213.9	832
57	Z	♀	4.13	1		0.77	173.8	676
58	Z	♀	4.14	8	39.5	0.76	184.5	715
59	Z	♀	4.21	9	39.8	0.78	211.8	811
60	Z	♀	4.27	4	39.6	0.74	201.2	765
61	Z	♀	4.39	4	39.2	0.74	180.1	672
62	Z	♀	4.42	4	39.7	0.72	157.1	584
63	G	♀	4.55	11	40.3	0.71	233.1	848
64	Z	♀	4.55	6	39.4	0.77	199.1	724
65	Z	♀	4.83	4	39.4	0.73	173.3	606
66	Z	♂	4.87	4	39.3	0.72	188.4	656
67	F	♂	5.16	3	39.5	0.71	239.6	801
68	Z	♀	5.17	8	40.1	0.70	180.2	603
69	F	♂	5.20	3	39.6	0.70	224.2	747
70	Z	♀	5.28	4	39.8	0.77	209.1	690
71	F	♀	5.39	1		0.75	243.2	792
72	F	♀	5.47	1		0.72	256.5	825
73	G	♂	5.67	1		0.71	275.9	868
74	F	♀	7.00	4	40.2	0.74	334.5	914

[1] Measured at 28° to 32°, between twenty-fourth and seventy-second hours of fasting; kept at thermic neutrality for 24 hours or longer before experiment.

[2] The breed is indicated by letters as follows: C = Chinchilla; D = Dutch; E = English; G = Checkered Giant; H = Himalayan; J = Japanese; P = Polish; Z = New Zealand. Where no letter follows the rabbit number, the breed is mixed or unknown.

[3] Average of the temperatures recorded at the end of each metabolism experiment.

[4] $S = 0.001w^{2/3}$; w = weight in grams.

ments accompanying the basal metabolism measurements were always within these limits. The heat production of individual rabbits with differing rectal temperatures within this range was carefully studied, but no consistent tendency was found among individual animals for high body temperature to be correlated with high heat production or vice versa. When the values for the different animals as a whole are considered, a correlation is noted between the heat production per square meter and the rectal temperature. The rectal temperatures were slightly higher and the heat production per square meter averaged appreciably higher at the heavier weights than at the lighter weights. How significant this correlation is, however, cannot be established by our limited data.

Size. The relationship between size and basal metabolism, already briefly discussed in the presentation of the basal data (p. 482), will be considered more in detail in a subsequent report.

SUMMARY

For basal metabolism measurements on normal adult rabbits, animals in good nutritive state with rectal temperatures between 38.4° and 41.1°C. should be used. The measurements can be made at any time of day or night. Absence of muscular activity is necessary. The rabbits should have fasted for from 24 to 72 hours prior to measurement, should have been living at environmental temperatures above 20° but not above 32°, and should have been habituated to the environment of thermic neutrality (28° to 32°C.) for 24 hours or longer prior to the observations at thermic neutrality. The initial experiment on any rabbit not previously accustomed to laboratory procedures should be considered only as an orientation experiment.

The rabbit's basal metabolism was established by 251 experiments on seventy-four adult animals, ranging in weight from 1 to 7 kg. The total heat production ranged from 60 to 335 calories per 24 hours and the heat production per square meter of surface area from 534 to 914 calories, being in general higher the heavier the weight. The males tended to

have a slightly higher metabolism than the females. There was no pronounced correlation between rectal temperature and heat production. The heat values per square meter were lower than most of the values reported in the literature, probably because of strict adherence to basal conditions of measurement.

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SIZE AND BASAL METABOLISM OF THE ADULT RABBIT

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TWO FIGURES

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The relationship of heat production to size among different animal species has frequently been studied, notably by Rubner (1883), Kleiber ('32), Brody ('34), and Benedict ('38), and various attempts have been made to correct for differences in size to bring the results for all animals to a comparable basis. This desirable end has not been fully attained. Even though differences in size may be to some extent ruled out by consideration only of various animal species of relatively the same body weights, there are numerous difficulties in comparing the heat productions of these animal species, such as differences in body configuration, body composition, and body covering. Thus far, the study of the factor of size per se has not been made under the most advantageous conditions. A better opportunity to study this factor is afforded by an animal species that has a large variation in the adult weights of various breeds within the species. The rabbit species is particularly suited for this study. It is possible to obtain rabbits having a sevenfold range in adult weights and yet, in spite of this large weight difference, having a configuration that is relatively unchanged (Castle, '30). Furthermore, the body composition is essentially the same in the large as in the medium and small-sized adult rabbits, as we have found from our analyses of the bodies of rabbits. Hence the difference in weight between the small and the large adult rabbit is not

a difference in the percentage content of body fat. In addition, domestic rabbits of all sizes live under practically the same conditions, having the same daily routine of feeding and activity, which is probably not true of many other animal species having as wide a range in adult weight. Therefore, for the study of the effect of difference in size upon metabolism the rabbit presents as nearly ideal conditions as are obtainable in any species. As the most practical index of size ordinarily available is the body weight, our consideration of size will be made with reference to body weight, and the terms 'body weight' and 'size' will be used synonymously. The discussion will be based upon our basal metabolism study of adult rabbits, the results of which have already been published (Lee, '39), and we shall seek to find some method of expression of the data by which the average trend of the heat production will be related to the weight as a simple function (or linear equation) or by which some direct relationship between the total heat production and the weight can be stated mathematically.

Total heat production referred to body weight. In figure 1 the values for the basal total heat production of our rabbits per 24 hours have been plotted with reference to the body weight. These data are the average results on each of seventy-four adult rabbits. Through the plotted values a curve has been drawn to indicate the general trend of the metabolism between the weights of 1 to 7 kg. Because of the variability in the metabolism of the different rabbits, the total heat production in a few instances is the same for rabbits differing in weight by as much as 1.5 kg. However, on the average, the total heat production between the weight limits of 1 to 7 kg. increases as a straight-line function of the increase in weight. In other words, the amount of increment in total heat production per kilogram of increase in weight above 1 kg. is the same at all weights. The percentage deviations of the measured heat production from the average metabolism as indicated by this curve have been calculated, and in table 1 the sum of the plus and the sum of the minus deviations (column c)

have been recorded for each weight group. For each group the difference between these two sums is small, which means that the straight-line curve has been drawn correctly to represent the average trend of the data throughout the entire weight range. The average total heat productions of the several weight groups, as calculated from the curve in figure 1 and as actually measured, are recorded in columns d and e of table 1. The close agreement between the measured and the calculated metabolism is further proof of the validity of the linear re-

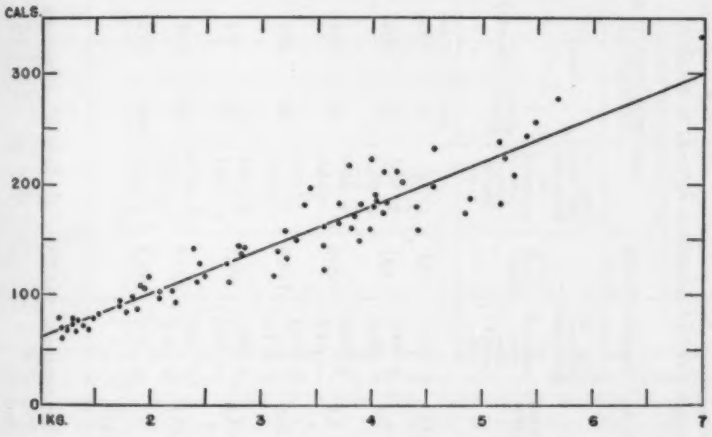

Fig. 1 Basal total heat production per 24 hours referred to body weight of rabbit.

lationship. According to the curve in figure 1 the average total heat production of the adult rabbit can be predicted by the formula $h_{total} = 39.35W + 22.5$, in which h_{total} represents total calories per 24 hours and W, the weight in kilograms. As the total heat production increases as a linear function of the increase in weight, the total heat production is related to weight.

Heat production per kilogram of body weight. If the average values for the total heat production at each kilogram of body weight as indicated by the curve in figure 1 are divided

TABLE 1

Comparison of the rabbit's measured heat production with that calculated on various bases
(Average trend of metabolism referred to weight)

WEIGHT (GROUP AND AVERAGE)	NUMBER OF RABBITS	TOTAL HEAT PRODUCTION PER 24 HOURS				PARABOLIC CURVE		HEAT PRODUCTION PER SQ.M. PER 24 HOURS				HEAT PRODUCTION PER $W^{0.73}$ PER 24 HOURS			
		Sum of per cent deviations of measured from calculated [1] heat	Average		Standard deviation	Sum of per cent deviations of measured from calculated [2] heat	Calculated heat [2]	Sum of per cent deviations of measured from calculated [3] heat	Average		Standard deviation	Sum of per cent deviations of measured from calculated [4] heat	Average		Standard deviation
			Calculated [1]	Measured					Calculated [3]	Measured			Calculated [4]	Measured	
kg. (a)	(b)	(c)	*Cals.* (d)	*Cals.* (e)	(f)	(g)	(h)	(i)	*Cals.* (j)	*Cals.* (k)	(l)	(m)	*Cals.* (n)	*Cals.* (o)	(p)
1.0 to 1.99 1.52	20	+ 66.8 − 67.1	82.3	82.6	± 8.4	+ 68.7 − 62.0	82.1	+ 65.1 − 66.3	625	624	± 8.3	+ 70.4 − 62.4	60.6	60.8	± 8.3
2.0 to 2.99 2.46	13	+ 47.8 − 51.0	119.3	119.3	± 9.6	+ 37.3 − 64.8	121.6	+ 44.8 − 54.9	658	654	± 9.6	+ 43.8 − 56.8	62.4	61.8	± 9.6
3.0 to 3.99 3.57	19	+114.6 −110.9	163.2	163.7	±14.8	+107.3 −121.2	164.6	+113.8 −113.2	699	699	±14.8	+113.0 −112.5	64.5	64.5	±14.8
4.0 to 4.99 4.33	14	+ 58.8 − 66.0	192.7	191.1	±11.1	+ 59.1 − 63.0	192.2	+ 58.4 − 66.1	726	721	±11.0	+ 60.9 − 63.0	65.9	65.8	±11.0
5.0 to 5.99 5.33	7	+ 30.2 − 30.7	232.4	232.7	±10.4	+ 39.8 − 26.3	227.9	+ 29.4 − 30.7	762	761	±10.3	+ 34.2 − 28.3	67.8	68.4	±10.6
7.0	1	+ 12.2	298.0	334.5		+ 17.8	283.9	+ 11.2	822	914		+ 13.8	71.0	80.8	
S. D. for all 74 rabbits					±11.3						±11.2				±11.3

[1] Calculated from curve in figure 1.

[2] Calculated from equation: total 24-hour heat production = $58.7W^{0.73}$ in which W represents weight in kilograms. See column e for measured total heat production.

[3] Calculated from curve in figure 2.

[4] Calculated from curve giving equation: heat production per $W^{0.73} = 1.9W + 57.7$.

by the respective weights, the resulting values for the heat production per kilogram per 24 hours are as follows:

Weight in kilograms	1	2	3	4	5	6	7
Calories per kilogram per 24 hours	61.9	50.6	46.9	45.0	43.9	43.1	42.6

Hence, as is generally recognized, a single value for heat production per kilogram of weight will not suffice to express the metabolism of rabbits of different weights, for the heat production per kilogram decreases rapidly at the lighter weights and less rapidly at the heavier weights. The value

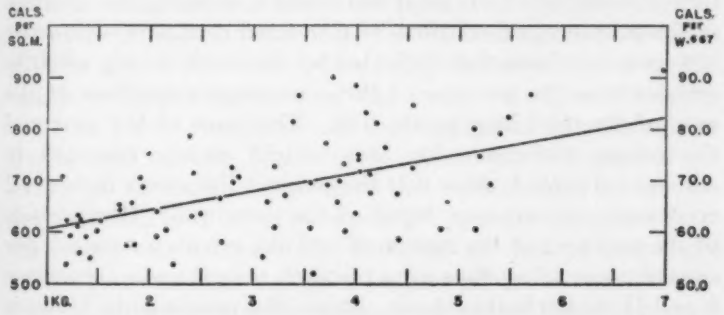

Fig. 2 Basal heat production per square meter of surface area per 24 hours referred to body weight of rabbit. The left-hand scale of ordinates represents the calories per square meter of surface area, obtained by dividing the total 24-hour heat production by $0.001w^{2/3}$, in which w represents the weight in grams. The right-hand scale of ordinates represents the calories per $W^{0.667}$, in which W is weight in kilograms.

at 7 kg. is 31% lower than that at 1 kg., and even between 1 and 3 kg., the weights most frequently found in the literature, the values decrease 24%. Therefore the change in the heat production per kilogram is not a simple function of the increment in weight, i.e., the relationship cannot be expressed as a linear equation.

Heat production per unit of surface area. In figure 2 the values for the average 24-hour heat production per square meter of surface area (left-hand scale of ordinates) have been plotted with reference to weight. The surface area was ob-

* Calculated from curve giving equation: heat production per $W^{0.73} = 1.9W + 57.7$.

tained by calculation, using the Meeh formula $S = \frac{K \times W^{2/3}}{10{,}000}$, where S is the surface area in square meters, W is the weight in grams, and K equals 10. Correction for the surface areas represented by rabbit's ears, which has been stressed by Rubner ('02), did not have to be made in our calculations, because our rabbits all had the same relative configuration and the present analysis is confined to the rabbit species alone. The curve in figure 2 indicates the average trend of the data. Although figures 1 and 2 give the impression that the scatter of the plotted heat values per square meter is much greater than the scatter of the total heat values, actually the average of the percentage deviations of measured heat production per square meter from that indicated by the curve is only slightly greater than the average of the percentage deviations in the case of the total heat production. The sums of the plus and the minus deviations for each weight group, recorded in column i of table 1, show that the straight-line curve in figure 2 represents the average trend of the data well. Comparison of the averages of the measured and the calculated values per square meter of surface area for each weight group (columns k and j) supports this thesis. Hence the relationship between the heat production per square meter of body surface and the body weight can be expressed by an equation of the first degree (a linear equation). In other words, the rabbit's basal heat production per square meter can be predicted from the formula $h_{sq.m.} = 36W + 570$, in which $h_{sq.m.}$ represents calories per square meter of surface area (calculated according to the formula given above) per 24 hours and W, weight in kilograms.

Heat production referred to powers of the weight. In the attempt to clear up the relationship between size and metabolism, Krogh ('16) has proposed expressing the heat production as a function of W^n rather than per square meter or any other unit of surface area, and he recommends that for warm-blooded animals n be taken, at least provisionally, as 2/3. The 0.73 power has been suggested by Brody ('34), Kleiber ('32), and the Committee on Animal Nutrition of the National

Research Council ('35). The individual total heat values for our rabbits were divided by several different powers of their body weights (in all cases expressed as kilograms) from 0.50 to 1.00 and plotted with reference to weight on each basis. For all the powers between 0.61 and 0.73, we consider that the general trend of the data is best indicated by a straight line, but for powers above and below these the median deviates from a straight line. Between the weight limits of 1 to 7 kg., the values for the heat production per $W^{0.667}$ are almost exactly one-tenth of the values per square meter of calculated surface area. Hence figure 2 (using the right-hand scale) shows the trend of the data per $W^{0.667}$ as well as per unit of surface area. For all powers of W between 0.61 and 0.73, the percentage deviations of the measured values from the medians are of essentially the same magnitude in the respective weight groups. In table 1, column m, the sums of the plus and the minus deviations for each weight group are given for the heat production per $W^{0.73}$, as an example. Since the 0.73 power is one of the limits of the powers showing a straight-line relationship between heat production per W^n and weight, the plus and the minus deviations for the several weight groups do not balance each other so well as is the case when the heat production is expressed with reference to other powers of the weight within the limits of the powers 0.61 and 0.73. The sums of the plus and the minus percentage deviations as recorded in column m of table 1 and the agreement of the calculated values with the measured values in columns n and o show that the straight-line curve correctly indicates for each weight group the average heat production per $W^{0.73}$. As this is also true for the other powers of W from 0.61 to 0.73, the heat production per W^n for values of n between these limits is related to weight by an equation of the first degree.

Total heat production as a parabolic function of the weight. As, theoretically, the heat production at zero weight would be zero, and as the basal total heat production of rabbits weighing from 1 to 7 kg. increases with the increase in weight, a

curve of the parabolic type passing through the origin or zero point would seem logically to apply to these data. The parabolic curve best fitting these data is indicated by the equation $h_{total} = 58.7W^{0.81}$ in which h_{total} represents the total 24-hour heat production and W, the weight in kilograms. In column g of table 1 are given the sums of the plus and the minus percentage deviations of the measured heat production from the metabolism as indicated by this parabolic curve. The difference between these two sums for each weight group shows that this curve does not fit the data so well as the straight-line curves. Other parabolic curves have been proposed for relating heat production to weight. Brody has proposed the formula $h_{total} = 70.5W^{0.734}$ and Kleiber, $h_{total} = 72W^{0.75}$. Neither of these conforms well to our data for the rabbit. Although at 7 kg. the heat values as predicted by the formulas of Brody and Kleiber are about the same as the average trend of the total heat production of our rabbits, at 1 kg. the values predicted by these formulas are 14% and 16%, respectively, above the average trend of our data at this point. However, the formulas of Brody and Kleiber are based on differences in size over a wide weight range, including many different animal species.

Comparison of the valid simple relationships of heat production to size. The rabbit's metabolism has been shown to have a linear relationship to weight when expressed as the total heat production, the heat production per square meter of surface area, and the heat production per W^n for values of n from 0.61 to 0.73. In all instances, however, the straight-line curves representing this relationship have an upward slope and are not horizontal. Hence, we have found no means of equalizing differences in the sizes of rabbits whereby a single value will accurately express, on the average, the basal metabolism of all rabbits. Whether such a constant relationship would be shown if the surface areas of our rabbits had been actually measured instead of calculated is a question that cannot be answered by our data. The measurement of the surface areas would probably result in a more exact factor for cal-

culating the area but probably would not eliminate the differences in heat value per square meter unless it happened that the factors varied according to increasing weights. The next question is, which straight-line curve will best express the rabbit's basal metabolism. When the sums of the plus and the minus deviations of the measured values from those calculated (for the same weight group) from each of the several straight-line curves are compared (recorded in columns c, i, and m of table 1 for three of the curves), the closest balances between the plus and the minus values in the several weight groups are those noted in column c for the total heat production. This signifies that the curve for total heat production expresses most accurately the relationship between the rabbit's heat production and its weight. In columns f, l and p of table 1 the standard deviations of the measured from the calculated metabolism, expressed as percentages, have been recorded for each weight group on the bases of total heat production, heat production per square meter of surface area, and heat production per $W^{0.73}$. The standard deviations are practically alike on all three bases for each weight group. For all seventy-four rabbits the standard deviations are also essentially the same on all three bases (see last horizontal line of values in table 1). Of all the relationships studied, although several were nearly as good, the best and likewise the simplest relationship found was the linear relationship between total heat production and body weight. The exact expression of this relationship is $h_{total} = 39.35W + 22.5$. For practical purposes, however, the equation $h_{total} = 40W + 20$ will serve as a more convenient method of calculation. With other animal species straight-line relationships between total heat production and increase in weight within the individual species have also been found, but the rates of increase in total heat production with increase in weight are not alike for all species (Benedict, '38).

Heat production of the rabbit compared with heat productions of other animals of the same weight. Comparison of the heat production of the rabbit with the heat productions of

other warm-blooded animal species of approximately the same weight is of general physiological interest. The comparison is best based on studies made by the Nutrition Laboratory, as, from the standpoint of technic, the conditions of measurement of the different animal species were thus the most uniform. The results obtained with the various animal species studied by the Nutrition Laboratory and by one or two other investigators have been summarized by Benedict ('38). The data for the rabbit given by Benedict include, in part, the basal values reported by Lee ('39) and, in part, the values reported by a few other investigators. Hence, Benedict's average for the rabbit is slightly different from the value given in this

TABLE 2

Comparison of total heat production of warm-blooded animals of like weight[1]

WEIGHT IN KILOGRAMS	CALORIES (AVERAGE) PER 24 HOURS							
	Rabbit	Wild birds	Marmot	Macaque	Goose	Hen	Cock	Cat
1	61.9	87.5						
2	101.2	130.0	62.5			112.0		100.0
3	140.6	172.5	81.0	167.0	173.5	146.0	157.0	152.5
4	179.9	216.0	100.0	199.0	222.5			
5	219.3	259.0		232.0	272.5			

[1] Data for rabbit derived from curve in figure 1; data for other animals derived from curves in figure 29 in the monograph of Benedict ('38).

present study. For the best comparison of the rabbit's metabolism with that of other warm-blooded animals of the same weight, the heat productions at different weights within the range from 1 to 5 kg. have been recorded in table 2 on the basis of total heat production. The values for all the animals except the rabbit have been obtained from the curves shown by Benedict in figure 29 of his monograph. For the rabbit, the values have been derived from figure 1 of this present paper. It is unfortunate that so few values for mammals are available at this weight range. One of the mammals, the marmot, is a hibernating animal, and its total heat production, even under normal, awake conditions, is much lower than that of other warm-blooded animals of like weight. Of the eight animals

represented in table 2, four are birds. It is generally recognized that birds have a higher metabolic level than mammals. Therefore, the comparison in table 2 is far from ideal. If it is legitimate to draw any conclusion from these few values, it can be stated that the rabbit has a metabolism slightly below the average levels of the other animals, except for the marmot.

On the basis of the average heat production per square meter of surface area (using for the rabbit figure 2 of this paper and for other warm-blooded animals of like weight Benedict's general curve for animals weighing from 20 gm. to 4,000 kg., given in figure 44 of his monograph), we find the following comparison:

Body weight, kg.	1	2	3	4	5	6
Calories per square meter						
Benedict's average	680	708	720	742	753	762
Rabbit	606	642	678	714	750	786

Here it is apparent that the rabbit's metabolism is below the general average at the lower weights but agrees with the general average at 5 kg. No attempt has been made in the above comparison to refine the calculations for the rabbit to correct for its relatively greater ear area as compared with that of other animal species, for we are concerned here not with the relationship between heat loss and surface area but with the relationship between heat production and size (weight).

SUMMARY

There is no known method of equalizing differences in the sizes of rabbits, which have a sevenfold range in adult weights, whereby a single value will accurately express the average basal metabolism of all rabbits. Within the rabbit species the relationship between basal metabolism and size is best expressed by referring the total heat production to the weight. For each kilogram increase in weight above 1 kg. up to 7 kg., the total heat production increases at a constant rate and can be predicted by the equation $h_{total} = 40W + 20$, in which h_{total} represents kilogram-calories per 24 hours and W, the weight in kilograms. Other simple linear relationships

between metabolism and size are found when the heat production is expressed per square meter of surface area and per W^n for values of n between 0.61 and 0.73, inclusive. The total heat production of the rabbit is slightly below the average metabolism of other warm-blooded animals of like weight, with the exception of the marmot. At 5 kg., its average heat production per square meter of surface area agrees with the general average for other warm-blooded animals, but at the lower weights it is below the general average.

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RESTORATION OF FERTILITY IN SUCCESSIVELY OLDER E-LOW FEMALE RATS [1]

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Several years ago it was noted in this laboratory that the usual 'curative' dose of wheat germ oil did not invariably lead to the birth of living young when administered later than the first half year of life to E-low female rats of proved sterility. This led to standardization of the test animal as a female under 6 months of age whose trial resorption gestation was begun at 60 days.

In the study herein presented, nearly 300 rats, ranging in age from 3 to 18 months, have been given graded doses of wheat germ oil. All rats were reared on the standard E-low diet 427 [2] and were bred for their trial gestation at the first

[1] Aided by grants from the Board of Research and from the Department of Agriculture of the University of California, from Merck and Company, Inc., and the Rockefeller Foundation, New York. Assistance was rendered by the Works Progress Administration, Project no. 10482-A5. The following materials were generously contributed: cod liver oil by E. R. Squibb and Sons, brewers' yeast by The Vitamin Food Company of New York, and wheat germ from which the oil was prepared by General Mills, Inc.

[2] Diet 427:

	%
Casein (commercial)	27
Cornstarch (cooked)	35
Lard	22
Cod liver oil	2
Yeast	10
Salt mixture no. 185	4

(The mixed diet without the cod liver oil was allowed to stand for 2 weeks at room temperature to permit the rancid substances of the lard to destroy incipient traces of vitamin E. The cod liver oil was added just before feeding.)

pro-estrus after the sixtieth day of age. The wheat germ oil was administered in a single dose by stomach tube on the day vaginal spermatozoa were encountered in the case of the groups receiving the lower levels of oil. In the group receiving 4 gm. of wheat germ oil, a 2-gm. dose of the oil was given on the first day and the remaining 2 gm. on the following day. In the case of the group on the 10-gm. level, five doses of 2 gm. each were given on alternate days.

Groups were bred at monthly intervals but in table 1, age groups which showed similar responses are combined. The

TABLE 1

Capacity of wheat germ oil to restore fertility to successively older female rats of proved sterility

WHEAT GERM OIL	AGE IN MONTHS	NUMBER OF COPULATIONS	PLACENTAL SIGN		NUMBER OF LITTERS	PER CENT LITTERING AFTER SHOWING PLACENTAL SIGN	AVERAGE NUMBER LIVING YOUNG PER LITTER
			Number	%			
gm.							
0.5	3–6	21	21	100	21	100	7.3
0.5	7–9	34	28	82	15	54	4.3
0.5	10–11	19	16	84	1	6	5.0
1.0	7–9	31	22	71	13	59	6.5
1.0	10–11	30	21	70	1	5	3.0
2.0	8–10	32	23	72	10	43	5.4
2.0	11–12	30	16	53	1	6	3.0
3.0	9–12	30	20	67	5	25	2.4
4.0	11–12	32	20	63	6	30	3.0
10.0	15–18	24	8	33	0	0	0.0

table shows that during the first half year of life, a single dose of 0.5 gm. of wheat germ oil invariably led to the birth of living young. An inferior outcome when 0.25 gm. wheat germ oil was fed has led us to designate 0.5 gm. as the minimal effective single dose (MED) of wheat germ oil for the restoration of fertility. The same dose, when administered from the seventh to the end of the ninth month of life, led to the birth of living young in only half the cases, and when given from the tenth to the eleventh month, was almost entirely inefficacious. A considerable elevation of wheat germ oil dosage

(two to four times) in the period elapsing from the seventh to the eleventh month does not substantially increase the instances of the birth of living young. Yet pronounced elevation of dosage does have an effect. From the eleventh to the twelfth month (a period, as has been mentioned, of virtually complete ineffectiveness of the MED of wheat germ oil) an eightfold elevation of the dose (4 gm.) led to the birth of living young in approximately a third of the cases (although the litter size remained subnormal).[3] From the fifteenth to the eighteenth month of life the administration of as much as 10 gm. of wheat germ oil did not lead to the birth of living young.

Until the end of the first half year of life, interference with implantation was not observed. In a total of 1211 test animals 3 to 6 months of age and of proved sterility, 94.7% have shown the placental sign following the next positive mating. This figure happens to be actually somewhat above the average for our stock colony (93%). This is not in agreement with the reports of Bacharach et al. ('37, '38) who state that they have found a clearly subnormal proportion of implantations in animals which had undergone a resorption gestation. Nevertheless, a comparison of the instances of copulation and the implantations following shows that there is evidence of increasing failure in implantation as age progresses. Established occlusion of the oviducts from infection explained about

[3] The possibility of increasing the number of instances of the birth of living young by fourfold dose increase of wheat germ oil was demonstrated by another group of twenty-two females on the E-low regimen but allowed to attain the ninth month of life without a trial gestation. Half of these received 1 gm., half of them 4 gm., of wheat germ oil on the inauguration of a test gestation. The results are subjoined. Here also the litter size was not improved by quadrupling the dose.

WHEAT GERM OIL	AGE IN MONTHS	NUMBER OF COPULATIONS	PLACENTAL SIGN		NUMBER OF LITTERS	PER CENT LITTERING AFTER SHOWING PLACENTAL SIGN	AVERAGE NUMBER LIVING YOUNG PER LITTER
gm.			*Number*	%			
1.0	9–10	11	10	91	4	40	4.5
4.0	9–10	11	10	91	7	70	4.3

half of the implantation failures, but we are not yet ready to state to what extent actual failure of the implantation mechanism accounts for the remaining half of such cases. In animals 15 to 18 months old no implantation sites were ever encountered, although uterine bleeding had been observed.

At autopsies, the uteri were invariably dark brown in color and were usually stiff and hard. Martin and Moore ('36, '38) found that rats (whether virgins or with the history of a single resorption gestation) when maintained for long periods on a vitamin E-low diet, developed a brown uterus which could not be restored to normal color either by treatment with a vitamin E concentrate or by shift to a diet consisting of natural food. Barrie ('38) found that the uterine pigmentation progressed with age. After 4 months on the diet there was a slight increase in the size of the uterus and definite, although slight, discoloration; after 5 months the discoloration and enlargement[4] were more marked and the condition was markedly expressed after 6 months. A resorption accelerated the appearance of the pigmentation. Barrie stated that the vitamin E requirement for restoring fertility increased as these changes advanced and postulated that pregnancy would be impossible if the uterine changes had progressed to an advanced stage.

We have given some attention to the phenomenon of uterine pigmentation. Twenty-seven E-low rats approximately 6 months of age were divided into three groups. Group 1 was maintained on the vitamin E-low diet throughout the experiment. Group 2 received 4 drops of wheat germ oil in addition to the vitamin E-low diet. Group 3 received 4 drops of wheat germ oil and were bred at the beginning of the experiment. All individuals were sacrificed at the end of 20 days. In agreement with the findings of Martin and Moore ('36, '38), the uteri were as darkly pigmented after the administration of wheat germ oil as in the control group, if the animals were

[4] The enlargement is not invariable. The average weight of the uteri and oviducts of fourteen of our E-low females 19 to 20 months old, with a history of two pregnancies, was 0.78 gm.; for eight natural-food virgin animals of the same age, the average figure was 1.31 gm.

not bred, but in the case of pregnant animals given wheat germ oil (in all cases living fetuses were found) the uterus was normal in color.[5]

In the present communication, we have contented ourselves with statements of fact and do not venture an explanation of the increasing reproductive disability of increasingly older vitamin E-low females. The need for elevated dosage with E shown by older E-low females can, we feel, hardly be taken to indicate that the embryos require more E with advancing age of the mother, but is preferably interpreted as due to: (1) increasing incapacity of the placental mechanism to deliver adequate vitamin E to the embryos, and (2) final failure of the implantation mechanism.

SUMMARY

The reproductive performance of successively older vitamin E-low female rats permits us to recognize three periods:

1. From the age of 3 to 6 months, at which time the response to a single dose of 0.5 gm. of wheat germ oil invariably leads to the birth of normal sized litters of living young. Only this period can be recommended for assay studies.

2. From the seventh to the twelfth month, at which time the response is variable; in the earlier part of this period only half the animals give birth to living young with this dosage (0.5 gm.) and these are undersized litters; moderate elevation of the dosage does not greatly change the result. Toward the eleventh month practically no animals respond to 0.5 gm. but even in this period great elevation of dosage shows a definite effect, for almost a third of such animals respond to 4.0 gm. of wheat germ oil, with living young but small litters.

3. After the fifteenth month of life no response can be secured with a twentyfold increase of dosage.

[5] Barrie ('38) observed a normal uterus in one instance of a female that had cast young.

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A COMPARISON OF CEREAL AND NON-CEREAL DIETS IN THE PRODUCTION OF RICKETS

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In a series of articles Mellanby [1] has presented evidence indicating that cereal grains contain a specific anti-calcifying factor. This evidence was based principally upon the observations (1) that there is a considerable variation in the rachitogenic properties of different cereals, (2) this difference could not be associated with either the total or relative amounts of calcium and phosphorus in the cereals studied and (3) the severity of the rickets produced was considerably decreased by a preliminary treatment of the grains with hydrochloric acid. Mellanby found that oatmeal produced more severe rickets than any of the other cereals investigated. Thomas and Steenbock ('36) in repeating some of the experiments of Mellanby, especially those of Green and Mellanby ('28) did not find a marked difference in the degree of rickets produced by corn, rolled oats, wheat and rice. Corn appeared to have slightly less calcifying action than the others. In an earlier publication Steenbock, Black and Thomas ('30) had observed that the equalization of the phosphorus content of various cereals by the addition of inorganic phosphates did not make the cereals of equal value in calcification. These authors suggested that all of the phosphorus from the various cereals may not be utilized to the same

[1] For references to most of the articles by Mellanby see the papers by Bruce and Callow ('34), and Green and Mellanby ('28).

degree. A considerable portion of the phosphorus in cereals is in the form of inositolphosphate which is incompletely hydrolyzed in the intestinal tract. Following the suggestion of Steenbock and associates, Bruce and Callow ('34) found that the addition of sodium inositolphosphate to a high calcium-low phosphorus rachitogenic ration was less effective in increasing calcification than an equal amount of phosphorus as disodium phosphate. Lowe and Steenbock ('36) later reported that the presence of liberal amounts of calcium carbonate further decreases the hydrolysis and thus the availability of inositolphosphate. Lowe, Steenbock and Krieger ('39) have recently reported that phytin phosphorus is less effective in increasing calcification in the chick than is an equal amount of inorganic phosphorus.

In all of these experiments cereals and cereal products have been used as the greater part of the diets studied and the conclusions have been based principally upon the comparison of one cereal with another. In an attempt to control the calcium, phosphorus and vitamin D content of the cereal diets other essentials frequently have been overlooked and inadequately supplied. Furthermore the grains themselves have been relied upon to furnish some of the necessary factors. Consequently, when one grain has been substituted for another it is possible that the diets being compared have differed in respect to factors other than those directly concerned with calcification. By using a synthetic diet and supplying these factors as individually purified substances the danger of any deficiencies in this respect could be eliminated. Also the effect of a variation in these factors among diets containing different cereals could be reduced to a minimum. The basal diet of Green and Mellanby ('28) was composed partially of purified ingredients. However, casein was used as the protein which precludes the possibility of having a diet low in phosphorus. In addition they supplied vitamin A by adding rather large quantities of dried cabbage which contains various dietary essentials including small and inconstant amounts of vitamin D. Furthermore, these authors did not compare diets con-

taining cereals with non-cereal diets which is possible when a diet based on individual components is employed.

In a recent report by the present author (Jones, '39) several practical, inexpensive and easily prepared rachitogenic rations composed of purified food materials were described. It was pointed out at that time, since severe rickets in the rat can be produced on purified diets it is improbable that cereals owe their rachitogenic properties to the presence of any specific anti-calcifying factor. In the present paper are reported the results of more detailed studies in which three cereals have been compared with each other and simultaneously with non-cereal diets in their ability to promote calcification in the rat.

EXPERIMENTAL PART

The rats used in these studies were placed on the experimental diet at approximately 25 days of age. They were kept in individual cages and except in one series of experiments daily food consumption of each animal was followed. After 21 days the animals were bled under ether anesthesia. The sera were analyzed for calcium by the method of Clark and Collip ('25) and for phosphorus on the calcium-free filtrate by the method of Gunther and Greenburg ('29). The right femurs were removed for the determination of the percentage of ash and the wrist bones for examination by the line test technic. The ash was determined on the dry, fat-free bone.

Several series of experiments were carried out. In each series five different diets were studied simultaneously with representative litter mate rats on each diet. Grains formed the principal part of three of the diets while the other two were composed primarily of purified materials. In the first series the basal diet consisted of alcoholic extracted fibrin 18%, yeast 5%, salt no. 5 (Jones, '39) 5.3%, agar 2% and carotene solution 0.1%. The diets were completed by adding respectively rolled oats, wheat, yellow corn, dextrinized cornstarch or sucrose to make 100%. The latter two diets are the same as rations no. 570 and no. 573 previously described

(Jones, '39). The particular grains were chosen because they were employed in some of the experiments referred to above and because of their widespread use in human and animal nutrition. Dextrinized cornstarch was used in order to include a comparatively pure cereal product. The ration containing sucrose was free of either cereals or their products. The total phosphorus content of these diets expressed in per cent is as follows: rolled oats 0.36, wheat 0.31, corn 0.26, dextrin 0.09 and sucrose 0.09. Phytin phosphorus was determined on the first three of these diets by the method of Harris and Mosher ('34) with the following results: oats diet—0.26%, wheat diet—0.22% and the corn diet 0.18%. The diets were all comparable in respect to calcium as 3% calcium carbonate was added to each. The results of this series as well as the following series of experiments are summarized in table 1.

A significant portion of the phosphorus in the above diets was present in the yeast. In the next series of experiments (series II) the phosphorus content of the grain diets was decreased by omitting the yeast which reduced the phosphorus in each case by about 0.05%. Grains do not contain sufficient quantities of all of the vitamin B factors for optimum growth in the rat in long-time experiments. However, it is generally considered that in experiments of short duration no marked deficiencies develop when any of the common cereal grains form the greater part of the diet. In the following experiments this apparently was true because growth on the grain diets was as good as on the non-cereal diets. The latter contained 5% yeast. The agar was also omitted from the grain diets of this and the subsequent series. This same experiment was repeated but the femurs were not analyzed for ash. Consequently, the results of the second experiment on these same diets are not included in the table, but, aside from the bone ash, they agreed with those presented in table 1 (series II).

Green and Mellanby emphasized the necessity of having a diet low in calcium in order to demonstrate the presence of the anticalcifying factor. In the next series (III) the same diets as in series II were fed with the exception that salt no. 10

at a level of 3.3% replaced salt no. 5. Salt mixture no. 10 is composed of the following ingredients expressed in relative parts by weight: NaCl 1, $CaCO_3$ 1, KCl 1, $MgSO_4$ (an-

TABLE 1

The comparative rachitogenic action of various cereal and non-cereal diets

DIET	NUMBER OF ANIMALS	DAILY FOOD CONSUMPTION [1]	GAINS IN WEIGHT [1]	SERUM [2]		FEMUR ASH [1]	
				Calcium	Phosphorus		
		gm.	gm.	mg./100 cc.	mg./100 cc.	mg.	%
Series I							
Oats	6	7.8	40.3	13.1 [3]	4.3 [3]	42.2	39.6
Wheat	6	8.3	37.4	14.1 [3]	4.3 [3]	28.5	33.2
Corn	6	7.9	28.8	11.9 [3]	2.8 [3]	17.5	23.1
Dextrin	5	5.5	20.0	12.7 [3]	2.1 [3]	13.8	20.2
Sucrose	5	5.8	20.8	12.8 [3]	2.7 [3]	19.2	26.1
Series II							
Oats	3	6.6	27.0	13.0	3.2	25.0	31.5
Wheat	4	7.2	26.2	12.7	3.3	28.6	31.0
Corn	3	6.4	15.3	12.5	2.8	16.2	21.0
Dextrin	3	6.8	26.7	12.0	2.1	19.5	22.8
Sucrose	3	7.1	24.0	12.5	2.2	21.9	24.6
Series III							
Oats	3	7.4	48.3	11.7	7.6	61.1	43.6
Wheat	3	8.7	48.0	12.1	5.9	53.5	41.5
Corn	3	7.8	36.3	11.8	4.4	35.3	34.7
Dextrin	3	7.9	33.3	13.2	3.7	25.2	25.8
Sucrose	3	6.8	27.0	12.7	3.9	18.7	23.2
Series IV							
Oats	3	5.7	21.3	5.8	9.3	34.1	41.2
Wheat	3	6.9	21.3	5.4	9.1	30.4	37.8
Corn	3	6.8	27.3	5.8	8.3	34.0	38.6
Dextrin	3	8.2	37.3	8.9	4.5	27.5	33.1
Sucrose	3	6.5	21.0	6.2	5.5	22.7	30.6
Series V							
Oats	3		7.3	5.6	11.1	31.3	37.9
Wheat	3		8.7	7.3	12.7	27.7	35.2
Corn	3		8.6	6.1	10.8	28.6	35.1
Dextrin	4		15.0	5.2	23.0	28.3	35.0
Sucrose	4		10.2	lost	lost	29.9	36.8

[1] Average of all animals on each diet.
[2] Analyses on pooled samples.
[3] Average of two pooled samples.

hydrous) 0.2, ferric citrate 0.1 and KI 0.004. Thus the diets used in series III differed from those in series II only in their content of calcium carbonate which was reduced from 3% to 1%.

In series IV no calcium was added; otherwise the diets were the same as in series II and III. The calcium content of these diets in per cent was as follows: rolled oats 0.08, wheat 0.08, corn 0.05, dextrin 0.06 and sucrose 0.06.

As a final series (V) the same diets as those of series IV were used except that phosphorus was added to each. This was done by feeding at a 4% level the Steenbock-Nelson salt no. 40 ('23) modified by omitting the calcium salts but keeping the phosphorus at the original level by the addition of disodium hydrogen phosphate. The amount of phosphorus added to each diet was approximately 0.5%. Food consumption records were not obtained in this series of experiments, but it was obvious that all the animals ate considerably less than in any of the other experiments.

DISCUSSION OF RESULTS

As seen from the results presented in table 1 there is a considerable difference in the degree of calcification produced on the various diets. In the first two series the diets were high in calcium and comparatively low in phosphorus. The cereal diets, however, contained considerably more total phosphorus than the non-cereal diets with the oats and wheat having a higher content than the corn. The degree of calcification, as judged by the bone ash and the concentration of inorganic phosphate in the serum, were considerably greater with the rolled oats and wheat diets than with the corn diet. In the latter case the results obtained were very similar to those on the non-cereal rations in which the total phosphorus was approximately one-third that of the corn. In the first series the gains in weight on the corn were intermediate between the two non-cereal diets and those containing either rolled oats or wheat. The consumption of the corn diet, however, was equal to that of the oats or wheat, whereas the food

intake on the non-cereal diets was somewhat less. As seen from the table this difference in food consumption between the cereal and non-cereal diets was not observed in the other experiments and thus cannot be considered as significant. In series II weight gains on the corn diet were the least of any of the groups, but with only three animals in each group not much importance can be attached to these differences. Food consumption was nearly the same for all groups in this series.

When the calcium carbonate was reduced to 1% as in series III the grain diets all produced considerably more calcification than either of the synthetic diets. In this case, with respect to their ability to produce calcification, the corn ration is intermediate to those of the other two grains and the non-cereal diets. The gains in weight were also less on the corn diet than on the other cereals although, as in the previous experiments, food consumption was about the same.

When no calcium was added as in series IV, the diets became essentially low calcium diets and the limiting factor for calcification was probably calcium and not phosphorus. As the calcium contents of all these diets were nearly equal, the degree of calcification expected would also be about the same. As the data show, this is true especially for the grain diets. The two non-cereal diets were very low in phosphorus and it is possible that in these cases phosphorus was still a limiting factor. The rather low levels of serum phosphate of the animals of these groups as compared with those on the grain diets are additional evidence that this was the case.

In series V sufficient phosphorus was added to all diets to assure an excess of this element. A deficiency of calcium was definitely the factor limiting calcification in each of these rations. The deficiency was of nearly the same degree in all groups and, as the data show, there was no apparent difference in calcification. Several of these animals showed definite tetany during the experiment which probably accounts for the very high phosphorus in the serum of the animals on the dextrin diet. Although this determination was not checked with a duplicate, we have observed at various times extremely

high levels of serum inorganic phosphorus during attacks of tetany.

The results of the examination of the wrist bones by the line test technic were in general in accord with the findings in other respects. Almost without exception the uncalcified areas at the metaphyses were wider and more regular on the synthetic diets than on the cereal diets. This was especially true of series III, although the difference was also very definite in series IV. In the latter series the metaphyses of the animals on the grain diets appeared practically normal but were considerably widened on the non-cereal diets. In the last series of experiments there was no widening of the metaphyses. As judged by this criterion in series I and II, corn was markedly more rachitogenic than either of the other cereals and wheat was slightly more rachitogenic than the rolled oats.

The above data are in accord with the hypothesis that the calcifying power of cereals is dependent primarily upon their contents of calcium and available phosphorus. Thus in the first two series of experiments the phosphorus was the limiting factor with corn containing less than either rolled oats or wheat. In series III, by reducing the amount of calcium carbonate a larger proportion of the phosphorus of the cereal diets became available, and their calcifying properties were definitely increased. The non-cereal diets, however, were still rachitogenic as the total phosphorus was low and, even if all absorbed, was not sufficient to prevent rickets. The difference in calcification exhibited by the animals on these diets therefore can be directly explained by a difference in the amount of utilizable phosphorus. In contrast to this, when phosphorus but no calcium was added to the diets, the extent of calcification was limited by the calcium. Inasmuch as all of the diets were nearly equal in their content of calcium, the degree of calcification would be expected to be approximately equal. Thus it is seen that when phosphorus is the limiting factor there is a difference in the calcification produced on the various diets because the amount of available phosphorus is not the same in each case. In contrast to this,

when calcium is the limiting factor and an excess of phosphorus is added, there is no appreciable difference in the calcification produced on the several diets because their calcium contents are all approximately the same.

SUMMARY AND CONCLUSIONS

Rolled oats, wheat, yellow corn, dextrinized cornstarch and sucrose have been compared with respect to their ability to promote calcification by feeding them as supplements to a basal mixture consisting of alcoholic extracted fibrin, carotene and a salt mixture complete except for calcium and phosphorus. With the calcium content of the diet high (3% calcium carbonate) and no phosphorus added, rolled oats and wheat definitely produced more calcification than did corn or either of the non-cereal rations. When the calcium carbonate was reduced to 1%, calcification on all of the cereal diets was considerably increased whereas there was no apparent increase associated with the dextrin or sucrose diets. Quite similar results were obtained when the calcium was omitted entirely, although the difference in calcification between the cereal and non-cereal diets was less.

When phosphorus was added to the low-calcium diets, there was no detectable difference in the calcification produced on any of the diets.

The above data are discussed in regard to the relation of the amount of calcium and available phosphorus in the diet to calcification in the rat.

No evidence was obtained in support of the view that cereal grains contain a specific anti-calcifying factor.

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THE DISTRIBUTION OF RIBOFLAVIN IN MEAT AND MEAT PRODUCTS [1, 2]

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The recognition of riboflavin as a specific member of the vitamin B complex necessitates a reappraisal of the available figures for its distribution in biological materials. Many of the earlier assays for the water soluble, heat stable vitamin were believed to be tests for only riboflavin, but they were, in reality, measures of more than one factor. Hoagland ('29) reported the antineuritic and water soluble B vitamin content of beef and pork. Hoagland and Snider ('30) estimated "the vitamin G in certain meats and meat products." The vitamin G in beef and veal has been determined by the rat growth method of Day ('31) and in the organs of lambs by Pierson ('34).

When crystalline riboflavin became available for standardization purposes, various spectrographic and colorimetric procedures were developed, thus providing additional figures for the occurrence of this compound in natural products. These methods have been criticized by Ellinger ('38). The crystalline riboflavin permitted the use of a definite standard in the biological methods which increased their accuracy. The values in the literature previous to 1934 on the distribution of riboflavin are not of a quantitative nature. In this paper we wish

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to report the amount of riboflavin in meats and meat products as determined by the microbiological method.

EXPERIMENTAL PART

These assays have been made by the bacteriological technic developed by Snell and Strong ('39). This method depends upon the growth of a specific strain of Lactobacillus casei on a media deficient in riboflavin. The basal media consists of 0.5% photolyzed, NaOH-treated peptone, 1% glucose, 0.01% cystine, a riboflavin-free yeast activator equivalent to 0.1% of the original yeast extract and a salt mixture. Five cubic centimeters of the basal media were pipetted into ordinary test tubes kept in a large metal rack. The supplement to be assayed and sufficient water to give a volume of 10 cc. were added to each tube.

The tissues assayed in this series were prepared for drying according to our previously described method and in many cases were the same samples as those used in our earlier work (Mickelsen, Waisman and Elvehjem, '39). The protein, fat and moisture analyses of samples 32 to 72 are given in the first paper, the values for the remaining samples are given in table 1. The riboflavin content of the material to be assayed was first roughly determined in a preliminary run so that a suitable weight of the sample could be used. As a result of this test, the kidney and liver were used at a level of 0.1 gm. and the other tissues at 1.0 gm. The accurately weighed tissue was placed in a test tube containing about 10 cc. of warm water. This was then homogenized in the apparatus described by Potter and Elvehjem ('36). The uniform mixture was then quantitatively transferred to a 50 cc. centrifuge tube and diluted to 35 cc. with water. These tubes were autoclaved at 15 pounds pressure for 15 minutes. As soon as the tubes cooled, they were centrifuged. The supernatant liquid was poured into a 100 cc. volumetric flask. The residue was suspended in about 20 cc. of water and centrifuged again. The washings were added to the original extract in the volumetric flasks. All extracts were finally diluted to

TABLE 1

Composition of some of the animal tissues used in these studies[1]

Meat	*Meat process*	*Meat sample*	*Moisture in original sample*	*Fat in dried sample*	*Protein in dried sample*
			%	%	%
Beef brain	Dried	77	...	45.3	44.6
Beef heart	Dried	73	78.7	8.4	73.6
Beef heart	Stewed	87	...	9.0	80.1
Beef kidney	Dried	81	77.2	11.5	73.0
Beef kidney	Dried	84	79.2	9.3	74.6
Beef kidney	Stewed	85	...	10.0	75.6
Beef kidney	Stewed	112	...	12.2	73.2
Beef liver	Dried	98	...	18.9	64.1
Beef liver	Dried	110	...	13.9	66.1
Beef liver	Fried	99	...	17.0	61.0
Beef liver	Fried	111	...	17.0	67.2
Beef lung	Dried	78	...	10.6	73.4
Beef pancreas	Dried	79	...	19.2	66.6
Beef pancreas	Dried	113	...	24.0	60.5
Beef round	Dried	105	72.3	12.8	71.6
Beef round	Fried	106	...	22.4	66.8
Beef round	Roasted	107	...	19.0	70.0
Beef spleen	Dried	76	...	9.0	66.3
Beef spleen	Dried	108	...	8.3	69.4
Beef spleen	Stewed	88	...	7.0	73.8
Beef spleen	Stewed	109	...	8.3	72.8
Beef tongue	Dried	82	72.2	42.4	49.4
Veal hindquarters	Dried	103	73.1	4.3	80.7
Veal liver	Dried	97	71.0	15.4	64.7
Lamb, leg of	Dried	80	74.2	14.0	74.3
Lamb liver	Dried	96	71.0	17.0	68.7
Pork ham	Dried	95	75.6	11.8	75.1
Pork ham	Dried	114	72.9	19.7	67.0
Pork ham	Dried	118	72.7	20.3	68.6
Boiled ham	Dried	101	67.1	12.2	61.6
Boiled ham	Dried	115	65.4	15.7	62.4
Smoked ham	Dried	102	71.5	11.6	59.3
Smoked ham	Dried	116	70.6	15.4	57.9
Tender ham	Dried	117	65.6	14.7	58.4
Tender ham	Dried	119	67.0	17.7	60.0
Pork heart	Dried	104	77.7	9.8	79.5
Pork kidney	Dried	83	78.1	13.2	73.0
Pork liver	Dried	86	67.8	9.7	54.1
Pork loin	Dried	74	71.8	24.4	66.2
Pork loin	Dried	89	66.6	33.4	59.5
Pork loin	Dried	90	71.4	9.7	81.6
Pork loin	Dried	91	70.0	24.4	63.6
Pork loin	Dried	92	70.1	34.5	56.0
Pork loin	Fried	93	...	32.6	59.6
Pork loin	Roasted	94	...	25.8	68.6

[1] For analyses of samples 32–73 see J. Nutrition, vol. 17, p. 272 ('39).

100 cc. and various aliquots of this solution were added to the basal media in the test tubes. In order to prevent the growth of microorganisms in the tissue extract, a small amount of chloroform was added to each sample. Control runs indicated that this and similar preservatives had no influence on the results of the method as long as care was exercised to prevent the addition of globules of chloroform to the test tubes. Usually, as a trial run, 0.5, 1.0 and 1.5 cc. of this solution were used. All assays were run in duplicate. In each series of assays standard tubes with graded levels of crystalline riboflavin were also set up. After the tubes were made up to volume, they were stoppered with cotton plugs and autoclaved for 15 minutes. Shortly after the tubes cooled, they were aseptically inoculated with a normal saline suspension of a freshly cultured Lactobacillus casei. The tubes were then incubated at 37° for 3 days. At the end of this period, the acidity in all tubes was determined by titration with N/10 NaOH. A reference curve was plotted from the titration values of the standard tubes containing the crystalline riboflavin. This curve was used in determining the riboflavin content of the unknown samples. Within a certain range, the amount of acid produced by the organism is directly proportional to the concentration of riboflavin in the media. All the work involved in the preparation of the extracts as well as the incubation was performed away from bright light so as to avoid a possible destruction of riboflavin in the aqueous solution.

Snell and Strong ('39) reported a series of assays on biological materials both by the bacteriological and rat growth methods. Both tests gave the same values for riboflavin which was conclusive evidence for the validity of the values secured by these assays. We have run similar parallel assays on some of our meats and meat products. The results secured by the rat growth and microbiological assays fully confirmed the previous results.

Simultaneous assays of the fresh and the dried material at equivalent levels showed that there was no destruction of

riboflavin during our drying process. A sample of pork liver (no. 122) was analyzed in both the fresh and the dried condition. The fresh sample was assayed by the bacteriological method shortly after the meat arrived from the abattoir. The same sample was also assayed as soon as it was dried. The fresh tissue contained 80 micrograms of riboflavin per gram on the dry weight basis, which agrees with the 85 micrograms per gram in the dry sample.

The results of the bacteriological assays (table 2) furnish additional confirmation for the high content of riboflavin in the livers and kidneys of all animals. Beef liver has varied between 105 and 125 micrograms of riboflavin per gram of dried material. Veal liver fell within the same range with a variation between 100 and 135 micrograms of riboflavin. Lamb liver, containing 90 micrograms and pork liver 80 to 90 micrograms per gram, showed a lower content of riboflavin. The dried beef kidney contained 90 to 100 micrograms per gram while pork kidney had 90 to 92 micrograms of riboflavin per gram. Next in potency came pork heart with 50 micrograms of riboflavin per gram while beef heart was slightly lower than this with 36 micrograms per gram. The pancreas of beef came next in order with 17 to 19 micrograms per gram followed by beef lung which contained 15 micrograms of riboflavin per gram. The remainder of the tissues including the muscular portions of the animals assayed contained approximately 10 micrograms of riboflavin per gram. One marked exception to this is the light meat of poultry, two samples of which contained only 3 micrograms of riboflavin per gram whereas one sample of dark meat contained 10 micrograms per gram.

In studying the influence of cooking on the riboflavin content of these tissues, a large cut of meat was secured and prepared according to our previously described method (Mickelsen, Waisman and Elvehjem, '39). One-half of it was cooked and then prepared for drying, whereas the other half was dried immediately. These studies revealed that stewing had no perceptible influence on the riboflavin content. The stewed beef kidney, stewed beef heart and stewed beef spleen

TABLE 2

The riboflavin content of meat and meat products

Meat	*Meat process*	*Meat sample*	*Flavin content /gm.* Fresh	Dry
Beef brain	Dried	57	2.5	12
Beef brain	Dried	77	...	11
Beef heart	Dried	73	7.6	36
Beef heart	Stewed	87	...	37
Beef heart	Stewed	54	...	34
Beef kidney	Dried	49	21.1	100
Beef kidney	Dried	81	22.8	100
Beef kidney	Dried	84	18.7	90
Beef kidney	Stewed	85	...	90
Beef kidney	Stewed	112	...	85
Beef liver	Dried	98	...	105
Beef liver	Dried	110	...	125
Beef liver	Fried	99	...	65
Beef liver	Fried	111	...	86
Beef lung	Dried	78	...	15
Beef pancreas	Dried	64	5.5	17
Beef pancreas	Dried	79	...	19
Beef pancreas	Dried	113	...	19
Beef round	Dried	40	...	9
Beef round	Dried	105	1.9	7
Beef round	Fried	42	...	6
Beef round	Fried	106	...	5
Beef round	Roasted	107	...	5
Beef spleen	Dried	59	3.5	15
Beef spleen	Dried	76	...	13
Beef spleen	Dried	108	...	16
Beef spleen	Stewed	88	...	13
Beef spleen	Stewed	109	...	15
Beef tongue	Dried	82	2.2	8
Veal hindquarter	Dried	56	2.4	10
Veal hindquarter	Dried	103	2.4	9
Veal hindquarter	Fried	45	...	11
Veal liver	Dried	70	...	100
Veal liver	Dried	97	39	135
Lamb, leg of	Dried	55	3.6	13
Lamb, leg of	Dried	80	2.8	11
Lamb liver	Dried	96	26	90
Pork ham	Dried	32	2.7	10
Pork ham	Dried	95	2.0	8
Pork ham	Dried	114	2.2	8
Pork ham	Dried	118	2.2	8
Boiled ham	Dried	101	2.6	8

TABLE 2 (*continued*)

Meat	*Meat process*	*Meat sample*	*Flavin content / gm.* Fresh	Dry
Boiled ham	Dried	115	2.8	8
Pork ham	Fried	52	...	9
Smoked ham	Dried	47	2.9	9
Smoked ham	Dried	102	2.3	8
Smoked ham	Dried	116	2.4	8
Smoked ham	Fried	48	2.6	8
Canned ham	Dried	72	2.2	7
Tender ham	Dried	117	2.4	7
Tender ham	Dried	119	2.0	6
Pork heart	Dried	104	11.2	50
Pork kidney	Dried	62	20	92
Pork kidney	Dried	83	19.7	90
Pork liver	Dried	86	29	90
Pork liver	Dried	122	26.9	85
Pork liver	Fresh	122	25.4	80
Pork loin	Dried	36	2	7
Pork loin	Dried	74	2.5	9
Pork loin	Dried	89	2.7	8
Pork loin	Dried	90	2.3	8
Pork loin	Dried	91	2.4	8
Pork loin	Dried	92	2.7	9
Pork loin	Baked	37	...	3
Pork loin	Fried	38	...	6
Pork loin	Fried	93	...	4
Pork loin	Roasted	94	...	3.5
Poultry, dark	Dried	66	2.6	10
Poultry, light	Dried	65	0.8	3
Poultry, light	Dried	68	0.7	3

had practically the same riboflavin content as the dried uncooked samples. When some of the samples were fried the riboflavin content varied considerably, with pork loin showing a destruction of approximately 50%. In the case of beef liver, the destruction was nearly 40% whereas about 33% of the vitamin was destroyed in fried beef round. The original uncooked samples were not available for purposes of comparison in the case of fried veal and pork loin but since a number of uncooked samples of these tissues contained nearly the same amount of riboflavin as the fried samples, it seems valid to conclude that there was very little destruction in both of these cases. Two cuts of meat were roasted. The beef

round after cooking showed a 30% destruction and the pork loin a 60% destruction of riboflavin. When pork loin was baked, about 60% of the original riboflavin was lost. It is difficult at present to explain the variation in the stability of riboflavin during different cooking processes. Further studies are being made in an effort to correlate the time of heating and the internal temperature of the meats with the stability of riboflavin.

DISCUSSION

From a comparison of the values for riboflavin in meats and meat products as reported in the literature and values secured by this method, it is evident that there is a close agreement in the results obtained with the rat growth and bacteriological methods. Darby and Day ('38) found 23.0 micrograms of riboflavin in fresh pork liver, while according to our procedure, there are 25.4 to 26.9 micrograms. These workers also found 2.8 micrograms in fresh lamb which agrees well with our values of 2.8 to 3.6 micrograms per gram. Similar agreement is apparent in the cases of cured and fresh hams. Charite and Khaustov ('35) by a colorimetric method found 35.6 micrograms of riboflavin in fresh calves liver whereas we found approximately 37 micrograms. Most of the values reported on the basis of the fluorometric method are considerably lower than ours. For instance, recent results reported by Schormüller ('39) are about one-half and in some cases only one-third of our values. The results reported by von Euler and Adler ('34 a, b) are also somewhat lower than those obtained in this study. The fluorometric method was used in both of these studies.

Sebrell and Butler ('38) have recently shown that certain patients, when restricted to the pellagra-producing diet of Goldberger, develop lesions around the mouth which are not cured by the administration of nicotinic acid but show a prompt response to pure riboflavin. This shows definitely that this vitamin is important in human nutrition. At present, no accurate figures are available on the human requirement. In 1937 Rose suggested that an adult should secure 20 Sherman-

Bourquin units of riboflavin per 100 calories. Since one Sherman-Bourquin unit is equal to 2.0 to 2.5 micrograms (Bessey, '38), the above value is approximately 1.0 to 1.2 mg. of riboflavin per day. Hogan ('38), in discussing Emmerie's work ('36) on the excretion of riboflavin in the urine of adults, concluded on the basis of the recorded data that the human adult required 2 to 3 mg. of riboflavin per day. The excretion studies were made on the assumption that all of the ingested riboflavin was absorbed and that none of it appeared in the feces or was utilized by intestinal bacteria, consequently these values may be slightly high.

On the basis of these values, we may assume that the human requirement for riboflavin lies between 1 and 2 mg. per day. The work reported in this paper conclusively shows that meats and meat products are an important source of this vitamin. The fried beef liver contained 65 and 86 micrograms of riboflavin per gram of dried material. In order to supply the suggested minimum daily requirement, approximately 12 to 15 gm. of dry liver or 50 to 60 gm. of the fresh material would be necessary. In the case of fried pork loin, approximately 450 gm. of fresh material would be needed to meet the daily demand for riboflavin.

SUMMARY

The riboflavin content of meats and meat products has been determined by the use of a microbiological method.

Liver and kidney of pork, beef, lamb and veal are uniformly higher than other organs of these species. The muscular tissues show a lower riboflavin content than the glandular tissues.

There appeared to be no loss of riboflavin by the ordinary household method of stewing. However, the samples that were roasted or fried showed appreciable losses.

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THE PRESERVATION OF THE 'GRASS JUICE FACTOR' IN SILAGE [1, 2]

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FOUR FIGURES

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In 1934 it was shown by Elvehjem, Hart, Jackson and Weckel ('34), and Stirn, Elvehjem and Hart ('35) that milk produced on a regular winter ration was markedly inferior in nutritive value to milk produced by cows on summer pasture. Kohler, Elvehjem and Hart ('36, '37, '38) have shown that various grasses contain a factor (or factors) which is essential for maintenance and growth of rats ('36, '37) and guinea pigs ('38). Small supplements of such grasses enable guinea pigs on a mineralized winter milk diet to grow normally.

Thus the seasonal changes in the nutritive value of milk can be related to the presence of a factor in the forage ingested by the cows; that is, during the summer the animals on pasture obtain sufficient 'grass juice factor' not only for body needs but also for transmission into the milk. It has been possible to decrease the growth-promoting value of milk at any time of the year by placing cows on a dry ration (winter ration) for several months.

It follows that by feeding forages rich in the 'grass juice factor' cows would produce a winter milk comparable to

[1] Published with the approval of the director of the Wisconsin Agricultural Experiment Station.

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summer milk in nutritive value. The problem then arises of preserving forages rich in the 'grass juice factor' for winter feeding. Since silage appears to be both a logical and practical means of carrying the factor, we have studied the preservation of the 'grass juice factor' in various types of silages. In this paper we wish to report the growth of guinea pigs on mineralized winter milk supplemented with various silages; and also on the mineralized winter milks from groups of silage-fed cows.

EXPERIMENTAL PROGRAM

Guinea pigs weighing approximately 300 gm. each were used. They were fed a basal diet of mineralized milk. The milk used was obtained fresh each morning from the same cow (Holstein) at the dairy barn of the university. This cow had been fed a winter ration low in the 'grass juice factor' for several months. Various kinds of silage plus a mineral supplement consisting of 1 mg. Fe as ferric pyrophosphate, 0.1 mg. Cu as copper sulphate, and 0.1 mg. Mn as manganese sulphate were fed. The silage supplement together with the mineral solutions was fed mixed with approximately 2 cc. of orange juice each morning. The orange juice was used to increase the palatability of the silage. Kohler, Elvehjem and Hart ('36) have shown that 2 cc. of orange juice daily, when added to mineralized winter milk, has no growth-promoting effect on guinea pigs.

In the afternoon, when the animals had completely consumed the supplements, enough milk was fed to allow ad libitum feeding. The animals were weighed each day and their weights recorded. Control guinea pigs were fed a diet consisting of the whole milk supplemented with the minerals.

Four kinds of forage were ensiled as follows:

(1) Alfalfa (June cutting) ensiled with 60 pounds molasses per ton.
(2) Alfalfa (June cutting) ensiled with 15 pounds phosphoric acid (79%) per ton.
(3) Clover and timothy in about equal parts (June cutting) ensiled with 60 pounds molasses per ton.
(4) Soy beans (October cutting) ensiled with 60 pounds molasses per ton.

Silages were also put up in milk bottles in the fall with clover, alfalfa, Kentucky blue grass and dried oat grass (dehydrated oat grass no. 1200-2 was supplied by the Cerophyl Laboratories). A. I. V., molasses and phosphoric acid silages were prepared from the fresh materials and A. I. V. silage from the dehydrated oat grass. A calculated amount of preservative in 50 cc. of water was added to 1 kg. of fresh plant material, and thoroughly mixed by hand. In the case of the dried grass, it was first mixed with water to form a paste. The amounts of preservative used per kilogram were:

(1) A. I. V. silage: 37.5 cc. of 2N acid (1 mol. H_2SO_4 + 1 mol. HCl); equal to 34 liters of acid per ton.

(2) Molasses silage: 32 gm. molasses; equal to 64 pounds per ton.

(3) Phosphoric acid silage: 15 gm. 79% H_3PO_4; equal to 30 pounds acid per ton.

The mixtures were packed tightly into milk bottles, closed with rubber stoppers containing Bunsen valves, and set aside. Each bottle contained approximately 750 gm.

At the time of cutting, samples of the soy bean, clover, alfalfa (third cutting) and Kentucky blue grass were prepared for feeding by drying 16 hours at 40°C. When dry they were ground and stored in the refrigerator. It has been shown by Kohler, Elvehjem and Hart ('38) that the activity of dried grasses disappears on storage at room temperature but that it is relatively stable at lower temperatures.

After the bottle silages had been allowed to stand for approximately 2 months, they were opened and the contents dried, ground and stored in the same manner as the fresh materials. Guinea pigs were fed these materials at a level of 3 gm. per day as a supplement to the basal mineralized winter milk. Because of the low 'grass juice factor' content of the dried fresh alfalfa (fig. 1) it seemed unnecessary to test the alfalfa bottle silages.

In figures 1 and 2 are given the growth curves of the guinea pigs fed the basal mineralized winter milk alone and plus the supplements of the materials which were fed both as dried fresh forages and as dried silages.

After about 6 months the University farm silages were sampled and tested in the same way as the bottle silages. The growth curves for these animals are given in figure 3.

The 'grass juice factor' content of the silage milks. The growth-promoting properties of milks from cows receiving the phosphoric acid alfalfa, the molasses alfalfa and the molasses clover-timothy silages were studied. Early in November,

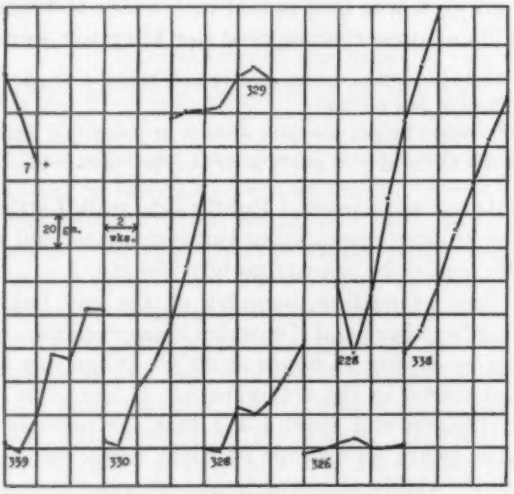

Fig. 1 Growth curves of guinea pigs fed mineralized winter milk plus supplements (3 gm. per day) of dried fresh forages and silages as follows: No. 7 control, no. 329 fall alfalfa, no. 228 dehydrated oat grass (fed at a 2-gm. level), no. 338 A.I.V. oat grass bottle silage, no. 359 Kentucky blue grass, no. 330 A.I.V. Kentucky blue grass bottle silage, no. 328 phosphoric acid Kentucky blue grass bottle silage, no. 326 molasses Kentucky blue grass bottle silage.

1938, three groups of cows were placed on experiment as follows: Groups 1 and 2 consisted of 1 Holstein, 2 Guernseys, 1 Jersey and 1 Brown Swiss; and in group 3 there were 2 Holsteins, 2 Guernseys and 1 Brown Swiss. The ration was made up of 42 pounds silage, 6 pounds alfalfa hay and 10.5 pounds grain mixture per day. Group 1 received the molasses

clover-timothy silage, group 2 the molasses alfalfa silage, and group 3 the phosphoric acid alfalfa silage.

Early in January, 1939 (i.e., 2 months after the cows had been placed on the silage ration), the feeding of the milks to the guinea pigs was started. All the milk from one milking of the cows in one group was mixed together and a quart aliquot taken. This was done for each group every day. Two

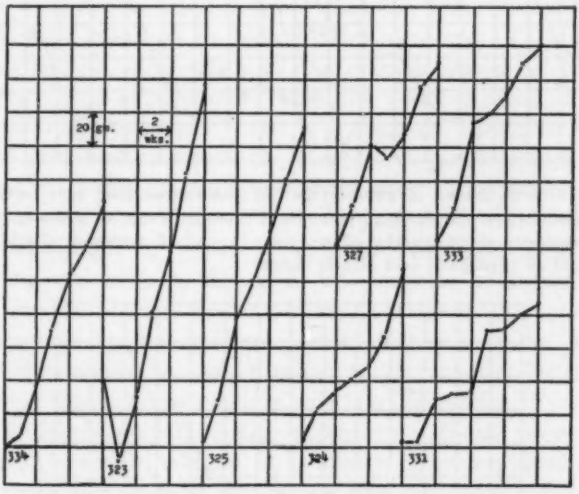

Fig. 2 Growth curves of guinea pigs fed mineralized winter milk plus supplements (3 gm. per day) of dried fresh forages and silages as follows: No. 327 soy bean, no. 333 molasses soy bean University farm silage, no. 334 red clover, no. 323 A.I.V. clover bottle silage, no. 325 phosphoric acid clover bottle silage, nos. 324 and 331 molasses clover bottle silage.

animals were fed each milk. They were fed a small amount of the milk plus the added minerals in the morning and after this had been consumed they were given an excess of milk. The growth curves of these guinea pigs are given in figure 4. Included for comparative purposes is the growth of a guinea pig on a good summer pasture milk, taken from the work of Kohler, Randle and Wagner ('39).

In order to determine if orange juice had any effect as used with the silage, one guinea pig was placed on milk from group 1 plus 10 cc. of orange juice daily. The curve of this animal (no. 17) is also plotted on figure 4. The pig died during the fifth week and showed gastric ulcers.

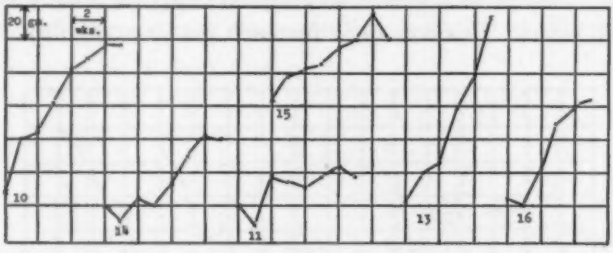

Fig. 3 Growth curves of guinea pigs fed mineralized milk plus supplements (3 gm. per day) of dried silage from the University farm as follows: Nos. 10 and 14 molasses clover-timothy silage, nos. 11 and 15 molasses alfalfa silage, nos. 13 and 16 phosphoric acid alfalfa silage.

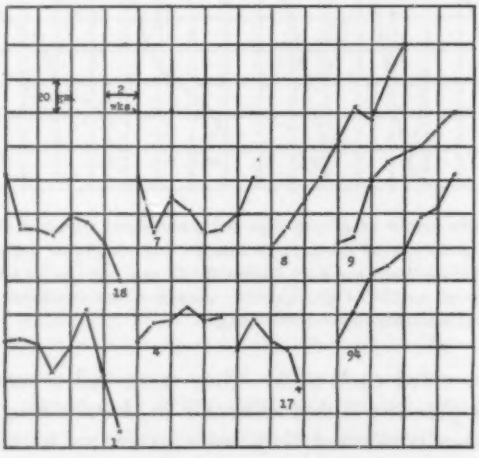

Fig. 4 Growth curves of guinea pigs receiving various mineralized milks as follows: No. 94 summer pasture milk, nos. 1 and 18 molasses clover-timothy silage milk, nos. 4 and 7 molasses alfalfa silage milk, nos. 8 and 9 phosphoric acid alfalfa silage milk, no. 17 molasses clover-timothy silage milk plus 10 cc. orange juice per day.

Animals 1, 2 and 4 (figure 4) after 6 weeks began to show loss of hair typical of a flavin deficiency and thereafter all the other guinea pigs on these milk diets were given a supplement of 50 micrograms of flavin per day. With this added flavin there were no cases of loss of hair and the weight seemed to be better maintained.

DISCUSSION

The results presented indicate that the 'grass juice factor' is definitely retained in plants after ensiling. This fact is obvious from a comparison of the growth curves of the animals on silage supplements with the control animal. However, it is obvious that there is a marked variation in the amount retained and that the degree of preservation is dependent upon the ensiling procedure used. It is evident that the acid-prepared silages were superior to the molasses-prepared silages in 'grass juice factor' content. Among the acid-prepared silages, the A. I. V. seems to be superior.

The reason for the lower potency of the molasses silages as compared to the acid-prepared silages may be due to destruction of the 'grass juice factor' during the initial fermentation which takes place in this type of silage.

Under farm conditions, fair retention is found even in the molasses-preserved silages, as is shown by comparison of the dried fresh and ensiled soy beans (fig. 2). Also the clover-timothy and alfalfa molasses silages gave some growth (fig. 3).

The potency of plant materials seems to vary with the stage of growth. The mature plants have been found much less effective than rapidly growing ones. The effect of maturity is shown by the good growth obtained on the young alfalfa cut in June (fig. 3) as compared to the poor growth obtained on the fall alfalfa cut in October (fig. 1) which was more mature. Ordinarily farmers allow their hay crops to reach a mature stage before harvesting, and at this stage the 'grass juice factor' content is low. This fact together with the destruction of the active principle during drying and storage accounts for the low potency of winter milk. However,

by acid ensiling at an immature stage, this factor is well preserved.

Comparison of various materials ensiled seems to indicate a very high 'grass juice factor' content for clover, young oat grass and for soy beans, a fairly high content for Kentucky blue grass and a lower content for alfalfa. It is difficult, however, to draw definite conclusions due to differences in state of maturity and time of harvesting of the various materials.

Figure 4 illustrates the fact that a winter milk high in the 'grass juice factor' was obtained by feeding the phosphoric acid alfalfa silage to the cows. By comparing guinea pigs no. 8 and no. 9 with no. 94 (fig. 4), it is seen that this phosphoric acid silage-produced winter milk is approximately equal to good pasture-produced summer milk. Comparing the growth curves on figure 4 with the control animal (no. 7, fig. 1), it seems that all of the silage-produced milks contain more of the factor than a dry ration produced winter milk. In this connection, it is interesting that Riddell et al. ('36) in studies on the vitamin C content of milk showed that 40 cc. of pasture milk, fed as a supplement to the basal scorbutic ration, produced much better growth in guinea pigs than did a similar supplement of milk produced by cows on a dry ration, and cows receiving silage produced a milk of intermediate growth-promoting qualities. Also Peterson, Bird and Beeson ('37) and Hegsted et al. ('39) found that rats fed mineralized milk produced by cows fed A. I. V. alfalfa silage grew more rapidly than rats fed mineralized winter milk and that there was no appreciable difference in the growth of rats fed milk produced from A. I. V. alfalfa silage, green alfalfa or pasture.

It is probable that the early observation that summer pasture milk was richer in vitamin C than winter milk was really a difference in the 'grass juice factor.'

SUMMARY

1. It was found possible to preserve the 'grass juice factor' in forages by ensiling.

2. The A. I. V. and phosphoric acid methods of ensiling were found to give better preservation of the 'grass juice factor' than did the molasses method.

3. Milk from cows fed phosphoric acid alfalfa silage was found to be approximately as rich in the 'grass juice factor' as milk produced by cows on summer pasture.

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